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MICRO-ORGANISMS AND FERMENTATION

MICRO-ORGANISMS

AND

FERMENTATION

BY

ALFRED JÖRGENSEN

DIRECTOR OF THE LABORATORY FOR THE PHYSIOLOGY AND TECHNOLOGY OF FERMENTATION AT COPENHAGEN

TRANSLATED BY

ALEX. K. MILLER, PH.D., F.I.C.

AND

A. E. LENNHOLM

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PREFACE.

THE present book gives an account of the morphology and biology of the micro-organisms of fermentation, and it thus forms a complement to the text-books which treat mainly of the chemical side of the subject.

I have attempted to give a *general review of all the knowledge we possess in this field*, and have described the various methods of investigation which in the course of time have proved of importance.

In discussing the organisms of fermentation and their relation to industry, there are two names which more especially attract our attention, namely, *Pasteur* in the older literature, and *Hansen* in the more recent literature of our subject. Since this book is intended to give an account of the *present stand-point of the science*, it is evident that the investigations from the Carlsberg Laboratory must occupy an important position. In Chapters V. and VI. will be found an accurate description of *Hansen's* theoretical investigations on the alcoholic yeasts, of his methods for the pure cultivation and analysis of yeast; likewise an account of the use of pure yeast on a manufacturing scale, and of the results obtained with it in breweries, distilleries, and pressed yeast factories, and in the preparation of wines from the grape and other fruits.

This book thus appeals to chemists, botanists, and biologists, likewise to those technologists who are engaged in the fermentation industries.

In the bibliographical list I have included all important works of both the older and more recent literature which are of interest to the scientist and technologist.

ALFRED JÖRGENSEN.

COPENHAGEN, *May*, 1893.

PREFACE TO THE THIRD EDITION.

A GREAT portion of this edition has been entirely re-written and considerably enlarged. Among its new features, I may mention a biological treatment, performed in my laboratory, of several English high-fermentation yeasts, isolated from yeast used in breweries and distilleries in various parts of Great Britain, and a summary of observations, made in my laboratory in the course of years, on the variations which yeast undergoes during its use in *factories*; further, descriptions of some particularly interesting yeast species discovered in recent years; and, finally, a concise account of the organisms occurring in milk, and of the use of pure cultures of lactic acid bacteria in dairies and distilleries.

These enlargements have necessitated the insertion of a considerable number of new figures.

Mr. S. H. Davies, M.Sc., has kindly revised, linguistically, all the new sections inserted in this edition. I therefore tender him my hearty thanks.

ALFRED JÖRGENSEN.

COPENHAGEN, *July*, 1898.

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MICRO-ORGANISMS AND FERMENTATION

MICRO-ORGANISMS AND FERMENTATION.

CHAPTER I.

MICROSCOPICAL AND PHYSIOLOGICAL EXAMINATION.

1. *MICROSCOPICAL PREPARATIONS, STAINING, AND MICRO-CHEMICAL EXAMINATION.*

THE **Microscope** will be for all time of paramount aid in the investigation of micro-organisms, since these, as individuals, are almost always invisible to the naked eye. The earliest important observations in the physiology of fermentation we owe to purely microscopical investigations, and it is only within the last few decades that biological and physiological investigations have been undertaken. After a certain probability had arisen that the same species of micro-organism did not always occur in the same form, work was eagerly commenced in different laboratories with so-called "*culture experiments*," in which attempts were made, by conditions of growth artificially brought about, to observe the different phases of development in *one and the same* spot, in order to determine thus the entire process of development. The idea was correct, but the way in which it was worked out at that time was so faulty that "culture experiments" threatened in consequence to fall into utter disrepute. The

work was carried out without any proper precautions, as the following example will show:

Beer yeast was sown on a moist slice of bread; the culture was carefully covered with a glass shade, and all manner of precautions were observed in order to protect the growth from *external* contamination. After some days a growth of mould appeared, as is always the case with moist bread; and the conclusion was therefore drawn that the beer yeast was the origin of the mould, and that, consequently, yeast and mould fungi were different phases of development of one and the same species.

A number of years elapsed before what are now universally acknowledged to be the obvious requirements of such investigations were put in practice, namely, that the first thing to be ascertained is the *point from which to start* before any conclusions can be drawn. This requirement was gradually defined with greater precision, and at last, as we shall see later, a point was reached which satisfies this demand in a higher degree than has hitherto been the case in the allied branches of science.

A microscope capable of magnifying to the extent of 1000 diameters is, as a rule, necessary for the investigation of micro-organisms. For the yeast and mould fungi the only preparation generally required consists in placing a drop of the liquid containing the organisms on an object-glass, and spreading it out in a thin layer by means of a cover-glass. When cultivated on solid substances, a very small portion of the growth is first mixed with a drop of water. At any rate, the preliminary examination of bacteria must always be performed in this manner.

In modern bacteriological research, and especially in the case of pathogenic forms, a number of different methods of *drying and staining* are employed, partly in order to facilitate observation, and partly with a view to bring out characteristics which would otherwise only be observed with difficulty or not at all. An objection to these methods, urged with unquestionable correctness, is that the violent treatment often alters the proportions of length, thickness, etc., of the bacteria. On the other hand, it may be alleged that certain pathogenic forms—

for instance, the tubercle bacillus, investigated by ROBERT KOCH,—could not be determined with certainty until such a preparation had been made; and, indeed, staining is often necessary in order to detect such bacilli. As an example of the methods of staining, we will enter somewhat more closely into the examination of the tubercle bacillus, which led to one of the most important observations made in modern science. KOCH gave the following method for its examination: The section of the tissue which contains the bacilli is immersed for 24 hours in a mixture of 200 parts of distilled water, 1 part of a concentrated alcoholic solution of methylene blue, and 0·2 part of a 10 per cent. potash solution. By this treatment the section is stained dark blue, and is then immersed, for a quarter of an hour, in a concentrated aqueous solution of vesuvine. The section is now rinsed in distilled water until the blue colour disappears, and a more or less intense brown stain remains; finally, the section is treated with alcohol, mounted in clove oil, and examined. The cell-nuclei and most species of micrococci are stained brown by this treatment, whereas the tubercle bacilli assume an intense blue colour. (Of the known species of bacilli, only the bacilli of leprosy behave in the same way; they differ, however, in other respects from those of tuberculosis.) According to KOCH, this result depends on the alkaline reaction of the staining solution, since these bacilli never take the stain in acid or neutral solutions; the neutral solution of another colouring matter entirely removes the first stain, except in the case of the tubercle bacilli, which retain the original staining. Subsequently, various other methods were proposed for the identification of this micro-organism, the most preferable of which is that of EHRLICH, who used aniline instead of potash. Aniline is a faintly yellow, oily liquid, the saturated aqueous solution of which has the power of taking up more colouring matter than the solution of potash. EHRLICH has also employed mineral acids for decolourising, proceeding on the supposition that the tubercle bacilli are surrounded by a cell-wall which is only permeable by alkaline liquids. Therefore, when the bacilli, cell-nuclei, plasma, etc., are stained by the alkaline solution, and the first-named are consequently practically indistinguishable in the

mixture, treatment with an acid removes the stain from all the other parts of the section and from all foreign organisms; but, as the presumed envelope of the tubercle bacilli cannot be penetrated by the acid, these bacilli will remain as the only stained bodies in the otherwise decolourised material. EHRLICH carries out the staining in the following manner: Finely-powdered gentian-violet is dissolved in a saturated aqueous solution of aniline; 10 to 20 drops of this solution are filtered into a watch-glass, in which the section to be examined is allowed to remain for about 24 hours. It is then rinsed with distilled water and again placed in the watch-glass with a solution of 3 parts of nitric acid in 100 parts of alcohol. After three to five minutes the section is decolourised; it is then transferred to pure alcohol, and finally examined in clove oil.

Another method of detecting these bacteria, which are of such frequent occurrence, was recently given by GABBET: Strong carbolic fuchsine is poured over the cover-glass and the latter heated by holding it for half a minute over a flame; then the fuchsine solution is poured off and replaced by a few drops of a dark-coloured solution of methylene blue in 25 per cent. sulphuric acid, which solution will in the course of some hours discolour and again colour the preparation. The latter is washed, and then examined in water.

As is well known, photographic illustrations of bacteria have recently come into general use, having been first introduced by KOCH. To obtain these, staining and decoloration are quite necessary, partly in order to render the contours of the bacteria sharper, and partly in order to remove all bodies detrimental to the picture.

Staining and decoloration are not generally required in investigations connected with the physiology of fermentation, where the organisms are almost always free, and seldom mixed with disturbing elements, and only in a few cases has staining led to the discovery of specific characters (Bacterium aceti and B. Pasteurianum, see Chapter III.).

On the other hand it is sometimes necessary in the examination of the organisms of fermentation, and especially of bacteria, to adopt another method of preparation. The

particles of organic and inorganic matter which separate from the solutions often have a deceptive *resemblance to various bacterial forms*; and, indeed, it is frequently a matter of the greatest difficulty, if not altogether impossible, for the most experienced observer to determine with certainty whether the small spherical bodies in the field of the microscope are micrococci or particles deposited from the solution. In such doubtful cases it is advisable, before entering on the physiological examination described later on, to have recourse to *micro-chemical reagents*, which often give good preliminary indications. In beer and in nutritive liquids generally which contain albuminoids, these often separate in spherical and thread-like forms; the starch granules, the dextrines formed from starch, and even some of the hop constituents may also appear as small spherical bodies. The addition of a small quantity of alcohol, ether, chloroform, acetic acid, soda, potash, etc., is often able to throw some light on the nature of these bodies, the resinous substances being dissolved by the former liquids, whilst the albuminoid matter is acted on more or less by the latter; the addition of iodine will impart a blue colour to the starch granules which are present, whilst certain dextrines are coloured red by the same reagent.

In the case of the higher organisms of fermentation—yeast and mould fungi,—staining is employed for a different purpose, namely, in order to obtain information concerning the *substances which are present in the cell-wall or cell-contents* at different stages of their development. On the addition, for instance, of a solution of ferric chloride, or any other salt of iron, to cells which contain tannic acid, a bluish-black or green coloration appears in the cells; in this way it was observed that the cells of Saccharomyces cerevisiæ contain a fairly considerable quantity of tannic acid during the earlier stages of fermentation. In certain conditions of the yeast-cells' existence a brown-violet coloration of the plasma is produced by iodine, which is interpreted as a glycogen reaction (compare Chapter V.). The so-called nucleus, which is directly visible in many fungi, is in others, and among them the yeast fungi, only observable if the plasma is fixed by means of quickly acting germicides (such as alcohol, picric acid) and then

certain staining reagents employed (as, for instance, hæmatoxylin solution).

2. *BIOLOGICAL RESEARCH BY MEANS OF THE MICROSCOPE; MOIST CHAMBERS.*

A true and thorough insight into the nature of the organisms of fermentation is not attainable until the *method of biological and physiological investigation* is resorted to. As stated above, endeavours were made long ago to devise methods of this nature; the entire neglect of precautions in carrying out the experiments resulted, however, in complete failure, and a reaction then set in, which found expression, *e.g.*, in the work of REESS on the Saccharomycetes (1870), in which he expressly stated that he had taken no precautions to obtain pure cultures,—to such a degree had these cultures fallen into disrepute. In the course of the following years, however, these methods were adopted under happier auspices, and microbiology has now reached a high degree of development. It is, perhaps, an almost unique fact in the history of science, that, in so short a time, a new method of investigation has not only been adopted, but has also yielded practical results, both in pathological science and in our own special branch, results which have brought about a revolution in many previously accepted doctrines.

The aim of biological and physiological investigations of micro-organisms is to gain an insight into their development and vital functions. The means to be employed in order to attain such an insight are, naturally, to determine such conditions for their growth and propagation as to make it possible to observe the changes gradually taking place in the organism itself and in the substances influenced by it. When the object aimed at is solely to obtain a *knowledge of the various forms* which the organism assumes during its development, the conditions are much more easily attained than when a culture on a large scale is attempted. Such a culture of individuals originating from one cell of the species is required for the purpose of gaining an insight, through *physiological, chemical,* or *purely practical experiments* with larger quantities of these

organisms, into the relations between their forms and external influences and into all their biological functions. In the former case all that is required is a culture in which the organism is able to develop itself undisturbed, apart from the question whether foreign individuals or species are present in the same preparation. In the latter case, on the contrary, an *absolutely pure culture is required.*

Cultures of the former kind may sometimes be of use in affording information in the case mentioned above; that of a nutritive solution in which deposits of various kinds have assumed a more or less deceptive resemblance to bacteria, in consequence of which it is impossible to obtain any certain information by means of an ordinary microscopical examination.

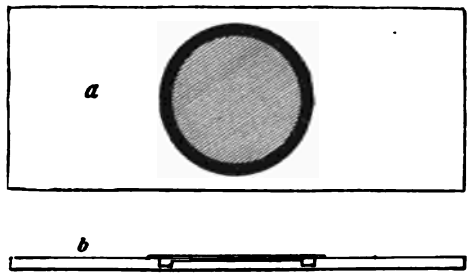

FIG. 1.—RANVIER'S Moist Chamber: *a*, plan; *b*, section.

The question to be answered by the experiment is therefore, whether these small bodies are *capable of multiplying.*

A drop of the liquid is transferred to the so-called *moist chamber*, as, for instance, RANVIER'S (Fig. 1). This apparatus is made by grinding a slight hollow in the middle of a common object-glass; round this hollow a groove is made of greater depth to receive the water. The drop of the nutritive solution, which must be very small, is placed in the middle of the hollow and covered with a cover-glass, which extends beyond the groove; when the cover-glass is in place, it is cemented by means of vaseline, and the drop is thus spread out between the cover-glass and the hollow of the object-glass, while the water in the groove makes it impossible for evaporation to take place.

Another kind of moist chamber, invented by BOETTCHER (Fig. 2), consists of a glass ring cemented to a common object glass, upon which, within the ring, some drops of water are placed. A cover-glass, on the under side of which a small drop of nutritive liquid containing the organisms has been placed, is fastened to the edge of the glass ring by means of vaseline.

This apparatus is brought under the microscope, and the changes of the organisms are observed from time to time; or it may be placed in an incubator, maintained at a suitable, constant temperature, and withdrawn at intervals for a thorough microscopical examination.

These forms of apparatus are adapted to morphological or botanical examinations under the microscope. If, on the other hand, a physiological examination is to be carried out, it is

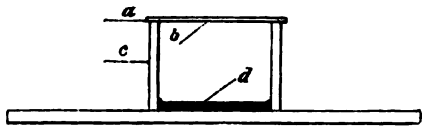

FIG. 2.—BOETTCHER'S Moist Chamber: *a*, thin cover-glass; *b*, layer of nutritive material; *c*, glass ring; *d*, water.

necessary that the pure cultures should be developed on an extensive scale. Among the investigators who have developed this line of work, PASTEUR, LISTER, KOCH, and HANSEN deserve special mention. (See "Preparation of the Pure Culture," § 7.)

Every fermentation, no matter whether the product be beer, wine, spirit, vinegar, or other liquid, is caused by a growth of living organisms, "organised ferments," and, in practice, our endeavour is to obtain, as far as actual circumstances will permit, a pure culture of the forms best suited to the manufacture. Although, in our time, with a better understanding of aims and means, great progress has been made in this direction, yet there must always be limits which, from purely practical reasons, cannot be surpassed; the cultures in the factories will probably never reach such perfection as to keep indefinitely in a state of absolute purity. It is, however, one of the most salient features in the present development of the industry of fer-

mentation, that efforts, based upon the right understanding of the paramount importance of the fermentation organisms, are being made to emancipate the chief useful species from the action of injurious forms. The very great importance of this was not, however, appreciated until HANSEN, through methodical selection of certain types of yeast, showed that such a pure growth insures far greater certainty and uniformity than the impure and unknown yeast mixtures hitherto used. We shall return to this point in Chap. VI.

In laboratory experiments, where the object is to prepare cultures of fermentation organisms, greater demands may naturally be made than on a practical scale. In this case it is necessary to work with *absolutely pure cultures*, first in small quantities, then in masses large enough to be transferred at a given time from the laboratory to the brewery. Conditions which are wanting in practice are realised in the laboratory, which is specially arranged for such investigations. We shall now briefly mention these requirements and the way in which they are met, and, for purely historical reasons, we shall begin with the final operation and describe the vessels and liquids which receive the previously prepared pure cultures, and the expedients to be employed in their cultivation. It is necessary that these vessels and liquids be *sterile* before the inoculating substance is introduced, *i.e.*, they must be freed from all living germs; also that the various utensils and the air in the place where the work is performed should contain as few living germs as possible. The same applies of course to the clothing and hands of the operator.

3. *STERILISATION.*

The principles of the technology of sterilisation as well as the models of the various apparatus appertaining thereto were described in the old memoirs on spontaneous generation.

As early as the year 1765, SPALLANZANI argued against the doctrine of NEEDHAM and BUFFON, that living beings came into existence through spontaneous generation in putrefying

liquids or other substances. SPALLANZANI warmed extract of meat in closed flasks, and demonstrated that the contents of the flasks remained unaltered until air is admitted. From this he concluded that the germs which developed in the opened flasks had come in from the air. Later (1782) SCHEELE demonstrated that *vinegar* may be prevented from decomposing by the application of heat. But his discovery was not heeded. In 1810 APPERT published his book on a method of preserving various foods and liquids by means of heat. In the fourth edition of his book, which appeared in 1831, he gives directions for the treatment of *wine, beer*, and other liquids, his method being essentially the same as that employed to-day (the so-called "Pasteurisation").

To the following period, which was of such great importance for micro-biology, belong the highly meritorious researches of F. SCHULZE (1836) and TH. SCHWANN (1837), in which it was shown that perishable liquids which had been vigorously boiled in flasks would remain sterile if the air subsequently admitted were made to pass through sulphuric acid or through red-hot tubes. At the same time MITSCHERLICH, CAGNIARD-LATOUR, SCHWANN, and KUETZING described the yeast cells, KUETZING also describing the acetic acid bacteria. TURPIN (1838) enunciated that most important doctrine: "No decomposition of sugar, no fermentation without the physiological action of vegetation."

The objection urged against the experiments of SCHULZE and SCHWANN, that the air entering the flasks had been affected in some manner by the violent treatment to which it had been subjected, so that it was no longer able to furnish the conditions of growth required by the germs existing in the liquid, was overcome by the beautiful experiments of SCHROEDER and DUSCH (1854), who obtained the same result by allowing the air to pass through cotton-wool filters.

Finally PASTEUR (1862) showed that, if in some experiments made by the last-mentioned investigators the boiled liquid still contained a growth of living organisms, it was owing to the substance *not having been boiled long enough.*

The principles of the whole technology of sterilisation being

thus established, the matter under consideration reached a high state of development and great importance both for science and for industry, due especially to the work of PASTEUR and, subsequently, to other eminent scientists, who devoted their energies to these investigations.

(*a*) Sterilisation of glass and metal articles.

Sterilisation properly so-called must always be preceded by a thorough mechanical and, in many cases, also by a chemical cleansing. Articles of daily use in the laboratory, as, for instance, spatulas, pins, wire, etc., are heated directly in a flame and allowed to cool in a germ-free space. Many pieces of apparatus, however, do not admit of this treatment, and must be sterilised either by heating in steam or in a water-bath, or else in dry air by means of a sterilising oven, in which the objects are heated for one or two hours at a temperature of about 150° C. According to the nature of the objects, some may be put directly into the sterilising oven, while others must be previously wrapped in paper. The necks of flasks are closed by cotton-wool, which should be covered by several layers of filter-paper.

(*b*) Sterilisation of nutritive liquids and solid nutritive substrata.

Nutritive liquids can be sterilised by filtration or by heat. The former method presents the advantage that the liquids treated undergo less change than when heat is employed, and are, consequently, better suited for the development of many species of micro-organisms. The necessary condition for momentary sterilisation is, that the pores of the filter must be smaller than even the smallest micro-organisms. Gypsum, asbestos, charcoal, porcelain, and other substances have been employed for this purpose, the liquids being forced by pressure or suction through thick layers of these substances. The forms most generally used are the Chamberland porcelain filter and the silicious mask filter, made of calcined infusorial earth; these filters, however, require constant cleansing, and must also be frequently sterilised, it having been proved that the bacteria

are able in time to grow through the pores of the filter, especially if the water contains much organic matter and the temperature is favourable.[1]

Liquids and solid nutritive substrata are in most cases sterilised by heat. The way in which this must be done, as well as the duration of the heating process, are dependent on the nature of the substratum in question. Direct boiling on the sand-bath may be employed for the purpose of sterilising, for example, brewers' wort in Pasteur flasks, or, again, the water-bath may be used. An excellent means of sterilisation is afforded by steam either at 100° C. or under pressure (110–120° C.) in a *Papin's digestor* (*autoclave*). During cooling, care must be taken that only absolutely pure air comes in contact with the sterilised substance, the air entering the vessel being filtered through cotton-wool or simply passed

[1] In breweries the **filtration of beer** has been resorted to during the last few years, the filtering media commonly used being paper, cellulose, asbestos, etc. By such filtration brewers sometimes succeed, it is true, in freeing a beer originally sound from deposits of various kinds, and in rendering it bright; but, on the other hand, the fact must be emphasised, that an indiscriminate employment of this method may *occasion great dangers*, as has been directly proved by the experiments made by THAUSING, WICHMANN, REINKE, LAFAR, and others. If the filters are not effective, it may happen that only the yeast cells are retained, but not the bacteria, which are then enabled to act with much greater energy upon the liquid. Another great danger lies in the fact that a filter, owing to deficient cleansing, may become a seat for the development of different kinds of germs, contaminating all the beer passing through it. If a single cask of a store-room has become infected, and the filter is not effectually *sterilised* after the filtration of this beer, the disease will be communicated to the whole of the beer.

The **filtering of water on a large scale** is commonly effected by means of *sand filters*, which are supported by several layers of stones of different size. In these filters a considerable portion of the bacteria are retained by the layer of mud deposited from the water covering the uppermost sand-layer. The water must therefore not move too quickly through the filter, otherwise the mud-layer will be broken up. In time, however, the bacteria grow into the sand, part of which must then be removed. The filter has been found to lose a considerable part of its power if the sand-layer is not at least two feet thick. The efficiency of such filters is always liable to fluctuations, particularly in consequence of changes of temperature and of the composition of the water, and, according to various investigations made in this direction, they cannot be expected to retain even a portion of the germs of the water for more than two or three weeks.

through tubes bent several times, if it is drawn in slowly and regularly.[1]

Nutritive gelatines must be treated with particular care, as they often lose their power of gelatinising if the heat is too great or if it is applied too long.

If the substance cannot be boiled without suffering great change or entirely losing its original nature, *fractional sterilisation* must be resorted to.

This, for instance, is the case with blood-serum, which is employed in a gelatinous condition in bacteriological studies. This substance, when heated to 100° C., becomes fluid, and does not again solidify, and it is, therefore, necessary to proceed in a different way in order to sterilise it in the gelatinous state. It was observed that a temperature of 58° to 62° C. in many cases sufficed to kill the vegetative bacteria which develop in blood-serum. By this treatment of the substance only the *spores* of bacteria remain unkilled. If the gelatinised serum is placed for two or three days in an incubator at a temperature favourable to the development of the spores (30° to 40° C.), a number of these germinate, and the new vegetative rods can then be killed by again heating to about 60° C. If this process is repeated several times, the gelatinous mass will commonly remain sterile for an unlimited time. This process, which is also used for the sterilisation of milk, and which was discovered by TYNDALL, has been further established by KOCH.

A similar method is employed in zymotechnical laboratories for the treatment of nutritive liquids, which, when boiled are apt to deposit a considerable amount of albuminoid matter, and

[1] In the so-called **Pasteurisation of beer, wine**, etc., a merely relative sterilisation is all that is generally aimed at; that is to say, by a cautious treatment of the liquid at elevated temperatures, it is attempted to check the yeast cells and other micro-organisms to such a degree that they are capable only to a very limited extent of multiplying and producing fermentation. It is only for transportation to a great distance or for preservation of the liquid for a very long time that the attempt is made to kill all living germs. *No general rules can be laid down for a treatment of this kind.* The correct procedure depends on the nature of the liquid as well as on the properties of the particular species of yeast, and preliminary experiments must always be made with regard to the temperature required and the length of time the operation must last.

would thus form comparatively poor nutritive media for the alcoholic yeast. According to the nature of the substance and the germs existing in it, this method requires different temperatures both for sterilising purposes and for the germination of the spores; and, as the result is uncertain, the substances thus heated should always be left for observation for a considerable length of time before use, to make sure that they are really sterile.[1]

(*c*) **Sterilisation of the air** is best attained, as stated above, by means of cotton-wool filters: sulphuric acid, brine, cloth filters, etc., are less efficient. In laboratories, where work must often be performed in germ-free air, a glass chamber is employed, the front of which can be raised sufficiently for the operator to introduce his hands. Some time before using the chamber, the whole of its inner surface must be washed, and the chamber then closed. The dust particles and germs suspended in the air will then settle to the moist bottom and remain there.

4. *DISINFECTION.*

Another method of killing disturbing germs is by the use of **disinfectants**, which act as poisons on the micro-organisms. Not a few of these substances have found application in practice. The limit for the employment of such poisonous substances must be determined for each individual case. As manipulations with such poisons may be deleterious to the

[1] Sterilisation is also attempted in practice for the purpose of *introducing brewers' wort in a sterile condition into the fermenting-tuns by means of a closed cooling and aerating apparatus.* It is true that the wort cannot keep absolutely free from germs when the fermentation takes place in open tuns, but a *great deal can be effected in this direction by acquiring a thorough comprehension of the matter.* The expert brewer will always take care that the air in the fermenting room is kept as free from germs as possible, by keeping the surface of all appliances clean, and also that the tuns as well as all utensils that are immersed in the fermenting liquid, *e.g.*, thermometers, sample glasses, etc., *are always perfectly clean. As a matter of course,* these precautionary measures could *not acquire any real practical importance until, through Hansen's reform, absolutely pure yeast had been introduced into the fermenting room.*

operator, it is important to ascertain their proper *degree of dilution.*

Investigations having for their object the determination of the *behaviour of the various species of micro-organisms to poisons* have proved that it varies greatly in different cultures of one and the same species, not only for the spores, but also for vegetative forms. A young culture behaves differently from an old one, and the same applies to individuals belonging to one and the same culture. In all experiments of this kind, organisms which have been tested with some disinfectant should afterwards be allowed the most favourable conditions of growth, otherwise they will not develop, even though they are alive and capable of development. In such cases, the ordinary temperature of the room and solid nutrient substrata are not sufficiently favourable: it is also necessary to allow ample time for the observation of such growths before definitely deciding whether they are dead or not; in fact, it often happens that they have merely been somewhat checked in their development, and that they may develop again, after some time, with their full vigour. Furthermore, the temperature and the medium in which the organisms are present when the disinfectant is employed may be of some importance. Before testing a culture thus treated, great care must be taken to previously free it from all remains of the disinfectant by washing and dilution.

As early as 1839, SCHWANN stated that yeast cells die under the influence of certain chemicals, the fermentation also coming to a stop. This discovery laid the foundation of the doctrine of antiseptics.

The first information on this subject we owe to ROBERT KOCH. These researches were continued by GRUBER and others.

KOCH examined several poisons not only with reference to the degree of concentration requisite for destroying bacteria and spores of bacteria, but also with a view of ascertaining the particular quantity necessary for checking the micro-organisms in their power of development in suitable nutritive solutions.

The results obtained by KOCH may briefly be summarized as follows: *Carbolic acid* was found to be a less efficient disinfec-

tant than it is commonly held to be. A solution containing 5 per cent. destroyed the power of development of anthrax spores only after 48 hours, whilst the anthrax bacilli were killed in two minutes by a 1 per cent. solution. A solution of 1 part in 850 proved sufficient to check the growth of the bacillus, and when anthrax spores were moistened 5 to 7 times with a 5 per cent. solution, their development was somewhat retarded. A 5 per cent. solution of carbolic acid in oil or alcohol had absolutely no effect on the anthrax cells and spores. In the form of vapour, carbolic acid acts more strongly, although even two hours' action of its vapour at 75° C. proved unable to destroy the vitality of these spores. *Sulphurous acid* is not able to destroy all germs, even under very favourable conditions. On the other hand, *chlorine*, *bromine*, and *corrosive sublimate* are efficient disinfectants. According to KOCH, corrosive sublimate in the proportion of 1 in 1000 acts fatally on all germs. According to experiments made by JOHAN-OLSEN, however, mould-fungi are only destroyed by more concentrated solutions; *e.g.*, Penicillium glaucum only by a solution containing 1 in 400. Several bacteria, the organisms of puerperal fever, abscesses, and putrefaction, likewise germinate and grow, although more slowly than usual, on slices of potato saturated with a solution of sublimate containing 1 part in 500, and their growth is only checked by a concentration of 1 in 300 under these circumstances. GRUBER found from recent investigations, carried out with all precaution, that anthrax spores, for instance, were only killed by 5 parts of sublimate in 1000, 1 part of sublimate hydrochloric acid in 1000, 1 part of sublimate tartaric acid in 1000. A disinfectant of common industrial use is *milk of lime*, which in a fresh state is very effective.

For cleansing pipes, coolers, etc., which often contain very considerable deposits of organic matter liable to decomposition through the agency of micro-organisms, a solution of *soda* is to be recommended; it acts by dissolving and loosening resinous and albuminoid matters, which can then be removed by means of water. The daily cleansing of conduits and india-rubber hose pipes must always be effected by *steaming*. Experiments made by AUBRY and WILL have proved that *chloride of lime*,

even when strongly diluted (2–5 per cent. of chlorine), is a very efficient disinfectant, owing to its deadly action on fungi. This substance being very cheap is therefore specially suited for cleaning walls, pavements, gutters, etc. *Bisulphite of lime* is also very efficient; that of commerce usually contains 7 per cent. of sulphurous acid, and is, when put into use, diluted with a two- or threefold volume of water. This substance is specially commendable for cleaning tuns and casks. The filter-bags—which, according to the investigations of WILL, often contain *very considerable accumulations of wild yeasts and bacteria in the very texture of the material*, and which in many breweries are never sufficiently cleansed—should be disinfected by treatment with a solution of chloride of lime. WILL, who experimented with material obtained from a brewery, recommends a solution containing 1 per cent. of active chlorine (corresponding to about 3–3½ kg. of good commercial chloride of lime to 100 litres of water). The aqueous mixture is stirred several times, and after deposition, the clear liquor containing chlorine is drawn off and put into use. The filter-bags must be washed with pure water after this treatment. In physiological laboratories, where it is of especial importance to guard against any invasion of foreign germs, an alcoholic solution of *salicylic acid* will prove of service (it is often used by HANSEN in the Carlsberg laboratory for cleaning tables). The action of this substance, in checking fermentation even in a diluted state, is generally known. In the case of very dirty tuns it is advisable to apply a very dilute alcoholic solution of salicylic acid, and, after some time, to rinse thoroughly, first with soda solution, and then with pure water. Recently, *hydrofluoric acid* has also been employed as a disinfectant.

Among substances which have recently come into use for this purpose, *formaldehyde* occupies a prominent place. This gas is prepared commercially as a 40 per cent. solution called *formaline*. Its power of killing bacteria is not inferior to that of sublimate, while at the same time it is less dangerous to the human system and that of the higher animals. The disinfection is effected in one of two ways: Cotton-wool or pieces of cloth are moistened with the solution and hung up in the infected locality; or specially constructed lamps are

used, in which the formaldehyde is produced by combustion of methyl-alcohol. Formaline is also used, in a solution of 1–2 per cent., for washing utensils, etc.

According to WINDISCH yeast-cells are less sensible to the action of formaldehyde than bacteria.

Very favourable results have also been obtained of late years by means of *antinonnine*, a creosote combination mixed with soap and other substances. It is, for instance, a powerful remedy for dry-rot, and may, according to the results of AUBRY, be used profitably in the fermentation industries for cleansing walls and utensils which do not come into contact with the liquids to be fermented.

In the manufacture of spirit and pressed yeast, researches have been carried on for some years past as to the **effect of antiseptics** on the **yeast-cell**, its power of fermentation, production of alcohol, and multiplying. Thus, HAYDUCK (1881) found that very slight quantities of *sulphuric* and *lactic acids* are capable of furthering both the fermentation and the growth of the yeast. In 1882 HEINZELMANN found that traces of *salicylic acid* increase the fermentative power, the yeast forming a larger amount of alcohol than otherwise within a given time. Later, BIERNACKI (1887) and SCHULZ (1888) found that all antiseptics under certain conditions, especially in minute doses, possess the property of accelerating and increasing alcoholic fermentation.—Of similar import are the recent statements of EFFRONT, that it is possible to stimulate the yeast-cell by using small quantities of *hydrofluoric acid*. According to EFFRONT, the addition of 0·3 grammes of ammonium fluoride to 100 c.c. entirely prevents the propagation of the yeast-cells, but does not destroy the fermentative power. It is possible, he says, by successive treatment, beginning with very small doses, to accustom the yeast to larger quantities of this poison, the amount varying with the selected species. First, the fermentation is introduced in 100 c.c. of wort with 20 mgr. of hydrofluoric acid; when half the sugar has been fermented, 100 c.c. of the fermenting liquid are mixed with 900 c.c. of wort containing 40 mgr. of hydrofluoric acid per 100 c.c. This procedure is continued till the last yeast is made to ferment in the presence of 300 mgr. of hydrofluoric acid per 100 c.c. of wort. In a

mash containing *ammonium fluoride* the yeast is then supposed to continue its vigorous fermentative activity. A necessary condition, without which such a treatment of the yeast cannot produce these results, is that a suitable type of yeast should first be found out by a methodical selection according to HANSEN'S principles. Finally, in this connection, HIRSCHFELD'S observation that acetic acid fermentation can be greatly accelerated by adding 0·01 to 0·02 per cent. of *hydrochloric acid* may also be mentioned. LAFAR'S observations as to the influence of *acetic acid* on wine-yeasts also furnish examples of such stimulating effects.

All these changes in the nature of the yeast-cells or bacteria are merely transitory.

5. *FLASKS: PASTEUR, CHAMBERLAND, FREUDENREICH, HANSEN, JÖRGENSEN, CARLSBERG FLASKS, ETC.*

All vessels in which cultures are made must satisfy the condition that they are proof to every contamination from without. **Pasteur's flasks** satisfy this demand in the highest degree.[1] The illustration (Fig. 3) shows this flask in the improved form employed in the Carlsberg Physiological Laboratory directed by HANSEN. When the hopped wort (preferably filter-bag wort) is boiled, the steam first escapes through the wide straight tube, at the end of which is a piece of india-rubber tubing; when this is closed (after boiling for about a quarter of an hour) the only outlet for the steam is through the bent tube. About ten minutes after, the flask is taken from the sand-bath, and the bent tube may be closed with

FIG. 3.—PASTEUR'S Flask.

[1] CHEVREUL and HOFFMANN had previously found that when vessels employed in sterilising liquids are provided with open but bent tubes their contents will remain sterile. Although CHEVREUL was thus the first to indicate the principle of these flasks, they will not be mentioned by any other name than that of PASTEUR, through whom, indeed, they have obtained a wide application.

a plug of asbestos. The contents of the flask can remain for years without undergoing any change. During cooling the air drawn into the flask is partially filtered through the asbestos-plug; the tube may, however, be open while the cooling process is going on; the germs are deposited in the lowest part of the bend, or, at the most, they do not pass the enlargement of the narrow tube, and therefore do not come into contact with the liquid. Hence, it is evident that the lower part of the bent tube must be heated whenever the flask is to be agitated or emptied through the straight tube, without exposing it to contamination. If the flask is to be opened and placed in connection with another flask, this must be effected either in some small germ-free space, or the opening and connecting must be done in a flame. A Bunsen burner is placed directly in front of the operator, the flask to be emptied to the *left*, and the one that is to receive the liquid or culture to the *right*, close to the burner. Then the tube of the left-hand flask is opened *in the flame* by quickly removing the india-rubber tube with its glass stopper: while the open tube is in the flame, the glass stopper of the flask to the right is quickly withdrawn, and the hot tube of the first flask is introduced into the india-rubber tube of the second flask. The liquid is now poured into the latter flask, the bent tube of the former flask being at the same time heated. Then the side tube of the left flask is again introduced into the flame, while the stopper of the right flask is heated and put back into its place; finally, the left flask is closed *in the flame* with its tube and stopper. When the operation is quickly performed, there is seldom any danger of contamination.

FIG. 4.—CHAMBERLAND Flask.

Pasteur flasks will be found indispensable in certain operations; as, for instance, in physiological researches where one has to deal with large quantities of liquids.

In recent years various other flasks and vessels have been brought into use, notably the **Chamberland flask** (Fig. 4), the

neck of which is closed with a ground cap, which terminates above in a short, open tube; this tube is filled with tightly-packed sterilised cotton-wool.

The **Freudenreich flask** is constructed on exactly the same principle; it has, however, a cylindrical shape.

For certain special purposes **Hansen's flask** (Fig. 5) is employed. The ground cap is provided with a cotton-wool filter (*a*), and the flask has a small side-tube closed with an asbestos

FIG. 5.—HANSEN Flask.

FIG. 6.

stopper (*d*). This flask is used partly for the preservation of pure cultures, partly for sending small cultures or samples from the propagating apparatus.[1] For the first-named purpose the flask is half filled with a 10 per cent. solution of cane-sugar, to which a trace of the yeast-culture is then added. The asbestos stopper and lower edge of the cap is coated with sealing-wax (*c*). For the last-named purpose the lower part of the flask is filled with cotton-wool (*e*), and some cotton-wool (*b*) is also put into the cap, below the filter. For the method adopted, see Chapter VI.

A modification of this flask has been constructed by the author (Fig. 6) by placing a small bent tube in the cap, as an immediate prolongation of the cotton-wool filter. By this means it has been possible to prevent the evaporation of the contents of the flask for several years, provided that the lower

[1] For the description of this apparatus, see Chapter VI.

edge of the cap and also the lateral tube are well closed. This

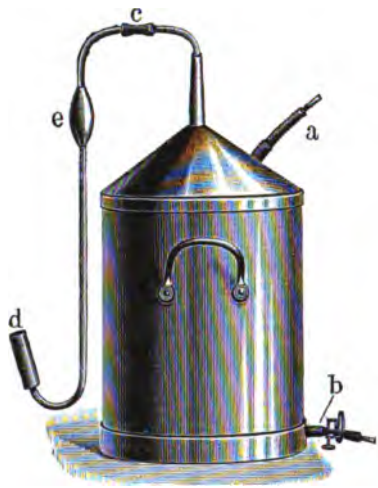

FIG. 7.—CARLSBERG Flask. Old Model.

flask is also suited to a prolonged preservation of gelatines, if these are to be prevented from getting hard on the surface.

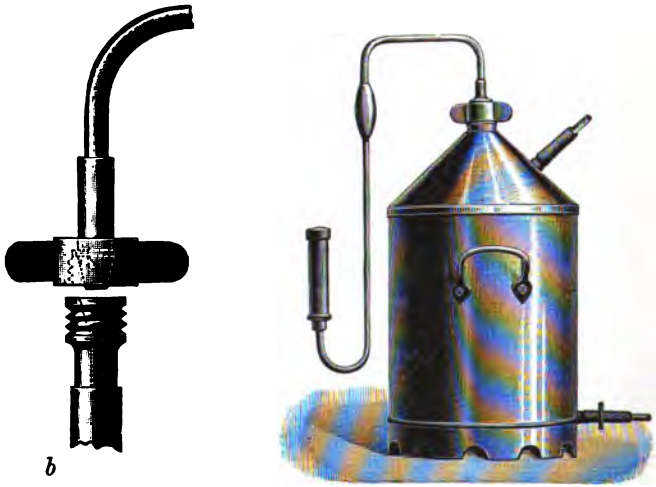

FIG. 8.—CARLSBERG Flask. New Model. *b*, connection between the flask and the bent tube.

For the development of very large cultures the Carlsberg vessels (Figs. 7 and 8) are employed. They have a capacity

of 10 litres, are made of tinned copper, are cylindrical in shape and conical at the top; at the apex of the cone a twice-bent tube (*c d*) with an enlargement (*e*) is either soldered or screwed. At one side of the cone is the inoculating tube and glass stopper (*a*), and at the bottom of the vessel is another tube (*b*) for drawing off the fermented liquid and the yeast. This tube is provided with a pinch-cock. When the liquid is sterilised, the bent tube is closed with an asbestos or cotton-wool filter, which is placed over the end (*d*).

In the new model (Fig. 8) the bent tube is ground into the upper part of the flask and held fast by means of a screw, allowing the whole of this part to be detached, when the flask is to be cleaned; the filter is also fixed with a screw to the point of the bent tube.

For further particulars respecting the treatment of these vessels, see HANSEN'S "Practical Studies in Fermentation."

6. *NUTRITIVE SUBSTRATA.*

With regard to the nutritive substrata, the problem naturally consists in finding those which are best suited to the respective organisms. If they also possess the advantage of being *per se* less favourable for the development of competing forms, it is a great point gained. The fact must of course be borne in mind, when comparative investigations are made in different directions, that the nutritive liquid must always remain the same. For the investigation of alcoholic ferments HANSEN generally uses hopped wort from the filter-bags; in special cases of investigations of this kind yeast-water with an addition of glucose, or a solution of cane-sugar or some other sugar, is employed. If it is desired to use a solid nutritive material, the liquid may be mixed with 5 to 10 per cent. of gelatine. Similar liquids, or more frequently, meat-extract with an addition of peptone, are employed for bacteriological investigations; this mixture is neutralised with sodium carbonate. Gelatine is used for rendering the medium solid. In case cultures are to be made at temperatures above 30°C., agar—a jelly derived from sea-algae—is used in the proportion of 1 to 2 per cent. For cultures of thermophilous bacteria,

say at 60 to 70°C., MIQUEL adds 2½ to 3 per cent. of carraghen to the nutritive liquid. Slices of potatoes are also used as solid nutritive media. For plate cultures of acid-forming bacteria some litmus or, preferably, carbonate of lime (fine, purified chalk) is added, by which means the colonies of these bacteria stand out clearly from those of other species, in that they appear to be surrounded by a clear, transparent zone. For certain bacteria silicic acid jelly is used as a medium. Solid nutritive substrata are the best for the study of mould-fungi, in most cases preferably sterilised black bread. Where liquids are employed, the most suitable are beer-wort, fruit decoctions, or mixtures of sugar with an addition of tartaric acid or tartrates. PASTEUR used liquids exclusively as substrata in his work on the organisms of fermentation. In more recent times, solid substrata have been very extensively employed, and in this respect KOCH has given many practical illustrations.

We have now briefly explained how our micro-organisms are cultivated, and guarded against contamination from the liquid itself, from the vessels and apparatus, from the air, and from the experimenter. We have now before us the first and most important question: *How are we to obtain the absolutely pure culture to be introduced into the flask?* We have, on purely historical grounds, first sketched the *conditions for the preservation of the pure culture*, because these were known long before a certain method for preparing the pure culture itself had been discovered.

In this respect it will be instructive to see how we have advanced step by step, and we will again take up the subject historically, from the moment when really rational endeavours were made to attain this object.

7. *PREPARATION OF THE PURE CULTURE.*

It is only by starting with one individual cell that we can be certain of obtaining a really pure culture, and such a culture is the indispensable condition for exact scientific investigations of the micro-organisms. These investigations may, as stated above, be carried out for different purposes, either with a view to observe *the individual, the isolated cell through its successive*

phases of development,—morphological investigation, or the object may be *to study the vital functions of a growth developed from a single cell,—biological and physiological investigations.* As these two *methods* of investigation are of different character, the means employed must likewise differ.

(*a*) **Pure cultures for morphological investigations.**—After the discovery had been made, by means of the microscope, that yeast consists of cells, it was not long before the attempt was made to determine, by closely observing one of these cells, the way in which they multiply, and in what forms the new generations occur. In other words, a morphological examination of a pure culture was made. For this purpose it became necessary to guard against such disturbances as would arise if other cells hindered the selected one from multiplying or concealed it from the observer's view. On the other hand, it would not matter if foreign cells occurred in other portions of the preparation.

EHRENBERG, as early as 1821, observed the germination of the spores of some fungi by careful observations of this kind. The propagation of yeast-cells was observed by MITSCHERLICH, KUETZING (1851), and F. SCHULZE (1860), in the same way. A small quantity of high-fermentation yeast was diluted with beer-wort until it contained only one or two yeast-cells; from a drop of this an ordinary preparation was made, the cover-glass was cemented fast on the glass slide, and the development of the cell was watched under the microscope. The same method was employed, in its main features, by TULASNE (1861) and DE BARY (1866), in their famous researches on the germination of the spores of the fungi. The investigation was carried further by BREFELD, who followed the development of the mycelium until it, in its turn, again formed spores. He sowed the spores on the object-glass. When his investigation was to extend over a longer space of time, during which an ordinary drop of liquid would evaporate, he added gelatine to the liquid, and placed a small shade of paper over the apparatus; this shade was attached to the tube of the microscope in order to keep out foreign germs as much as possible. When the development took place in ordinary fluid drops, the preparation was placed in the interval between two

observations, under a moist glass-shade; thus, an unbroken observation was not attempted, and was not even possible for the larger fungi. It follows from the whole arrangement of the experiment, that absolutely pure cultures were out of the question. As stated above, however, such an investigation may well be carried on with impure material.

(*b*) **Pure cultures for biological and physiological experiments with mass-cultures.**—When the object of the pure culture is to employ it for *biological* or *physiological* researches, so that a *mass-culture* of the growth becomes necessary, a direct microscopical control of the whole culture is impossible, and the methods described above cannot be employed. The methods made use of for this purpose may be divided into two groups, namely, the *physiological methods* and the *dilution methods.* In the former liquids are used, in the latter liquids or gelatines.

(*a*) **Physiological methods.**—The physiological methods employed by PASTEUR, COHN, and others, start with the fundamental idea, that the various species occurring in a mixture will multiply unequally according to their different natures, when they are cultivated in one and the same nutritive liquid and at the same temperature, so that those species for which the conditions are unfavourable will be gradually suppressed by the one or more species for which the conditions are favourable. Different liquids have been employed for such cultures from time to time; as, for instance, alkaline liquids for growths of bacteria, acid liquids for the purpose of freeing yeast-growths from bacteria (lactic, tartaric, hydrofluoric acids, etc.). The weak point of all such methods is, that they *start from an unknown material,* namely, *the impure mixture.* Hence, it is impossible to know what results a treatment of this kind will lead to, because it is evident that any agency exerted will be hap-hazard, and this does not, properly speaking, constitute a method. In fact, there is always the possibility that the weaker species are not destroyed at all, but merely checked and retarded, so that when the stronger species, after having reached the height of their development, enter into a condition of weakness, other species will have a chance of multiplying. Likewise, there is always the

possibility that not one but two or more species thrive equally well in the liquid, and, consequently, develop to the same extent. Such, for instance, was the case with brewers' yeast before pure cultures were employed. This yeast often yielded several typically different species of "culture-yeast," as they are termed, when examined by HANSEN'S method. The method given by PASTEUR for the purification of brewers' yeast may be mentioned as a marked illustration of the dangers connected with the physiological method of treatment. The impure yeast-mass is introduced into a cane-sugar solution to which a small amount of tartaric acid has been added. The object of the method is to free the yeast from any disease germs with which it may be infected. HANSEN'S investigations have, however, proved that, even if the bacteria are suppressed or checked by this treatment, the so-called wild yeasts, and among them *those productive of diseases in beer*, will develop abundantly, and in many cases the culture-yeast becomes totally suppressed instead of being purified. Even if there is primarily only a trace of the wild yeasts or "disease" yeasts, these are apt to develop to such an extent during this treatment that they may eventually form the chief portion of the yeast-mass. Thus, this unmethodical treatment of the unknown material has led to an exactly opposite result to that intended. Even when the yeast-mass consists entirely of the so-called wild yeasts, it is not possible by this process of Pasteur's to prepare with certainty a pure culture of a definite species.

The use of *hydrofluoric acid* or its combinations, such as ammonium fluoride, for the purpose of purifying an impure yeast—brewers' or distillers' yeast—as proposed by EFFRONT, is liable to lead to the same dangers as the use of tartaric acid described above. Methodical experiments made by HOLM and the author have shown that by treating impure yeast according to EFFRONT'S directions, the growth of *wild yeast* and *Mycoderma species is forced more than that of the culture-yeast*; they have also shown that such a dangerous species as *Bacterium aceti* is in many cases not suppressed at all by the treatment in question, but, on the contrary, *multiplies more* rapidly in presence of hydrofluoric acid or fluorides.

If, now, we ask, whether it is advisable to employ any of the various methods mentioned above for the purification of an unknown and impure yeast-mass, the answer must be in the negative; and this will be the case whether the culture is intended for purely scientific or for industrial purposes, for the danger will always remain of *forcing the growth of species other than the desired one.* The starting-point being uncertain, it necessarily follows that the result must be so too. In fact, all such methods must now be regarded as antiquated, and will, whenever resorted to, prove utter failures. Nevertheless, they may possibly be used in certain cases of rare occurrence before proceeding to the preparation of a pure culture. In the different branches of the fermentation industry there is but one way that will lead to the goal, namely, the application of the same principles which have for many years been followed in agriculture and horticulture—the *selection, by means of methodical experiments, of the particular species or type* which gives the best results under the existing circumstances, and which is therefore to be sown alone, without admixture of other types. The only possible way of effecting this is, however, by the adoption of the methods discovered by HANSEN, which will come under consideration later on.

(β) **Dilution methods.**—The second group of methods employed for physiological purposes embraces the dilution methods, or the so-called "fractional cultivation," the principle of which is to dilute the material to such a degree that it is ultimately possible to isolate a single cell. In most of these cultures we can only reckon on their probable purity, whereas for the alcoholic ferments HANSEN has developed the process into an *exact method.*

LISTER (1878) was the first to introduce methods of this kind. In order to prepare pure cultures of lactic acid bacteria he first determined microscopically the number of bacteria in a very small drop of sour milk, counting them in several fields of the preparation, and thus calculating their number in the whole preparation. He then estimated the amount of sterilised water it was necessary to add so that after dilution there would be on an average less than

one bacterium in each drop. With five of these drops he inoculated in one case five glasses containing boiled milk. The result was that the milk in one of these coagulated, showing that it contained Bacterium lactis, whilst the four other glasses remained unaltered and did not show the presence of bacteria. The same method was subsequently employed by NAEGELI and FITZ.

Air has also been made use of for such a dilution (PASTEUR). A small portion of yeast is dried and ground with powdered gypsum. The resulting fine powder is thrown into the air from a height, a series of vacuum flasks being opened while the particles are falling. Isolated yeast-cells which are distributed in the resulting dust-cloud may possibly enter some of the flasks.

In comparison with the physiological methods the dilution method now described is a distinct advance; indeed we have thus approached much nearer to the goal. On the other hand, it is clear that, even if the dilution is carried as far as in the case mentioned, in which only one of several flasks shows development, it is not yet proved that this one flask has received only *one germ*. Thus, there is still great uncertainty, even in cases where the individuals with which we are working can be counted. Moreover, it is difficult to count individual bacteria, and often, indeed, quite impossible. In all cases the accuracy of such calculations is very questionable. Thus, the problem remains to be solved: How are we to distinguish the flasks which have only received *one cell* from those which, in spite of the counting, have been infected with *several cells*? For the bacteria, no means have as yet been found of solving this difficulty.

In the case of yeast, this problem was solved by HANSEN, who developed the method to such a degree of perfection as to create, in fact, an *exact method* (1881). He employed dilution with *water*, in the following manner:—The yeast developed in the flask is diluted with an arbitrary amount of sterilised water, and after vigorous shaking, the number of cells in a small drop of the liquid is determined. The counting, in this case, is effected in a very simple manner by transferring a drop to a cover-glsss, in the centre of which some small squares are en-

graved, and this is then connected with a moist chamber (Fig. 2); the drop must not be allowed to extend beyond the limits of the squares. The cells present in the drop are then counted. Suppose, for instance, that 10 cells are found; a drop of similar size is transferred from the liquid, which must first be shaken vigorously, to a flask containing a known volume, *e.g.*, 20 c.cm. of sterilised water. This flask, then, will in all likelihood contain about 10 cells. If it is now vigorously shaken for some time until the cells are equally distributed in the water, and then 1 c.cm. of the liquid introduced into each of 20 flasks containing nutritive liquid, it is probable that half of these 20 flasks have received one cell each. But, here again, as in LISTER'S experiments, it is entirely a calculation of probabilities. If the flasks are allowed to stand for further development of micro-organisms, there will be a chance of getting a pure culture in some of them. But no certain inferences can be drawn. HANSEN succeeded, however, in adding a new factor, which first gave certainty to this experiment. Thus, if the freshly inoculated flasks are vigorously shaken, and then left in repose, the individual cells will sink to the bottom, and be deposited on the wall of the flask. It is self-evident that if a flask contains, for instance, three cells, these cells will always, or at least in the great majority of cases, be deposited in three distinct places on the bottom. After some days, if the flask is raised carefully, it will be observed that one or more white specks have formed on the bottom of the flask. *If only one such speck be found, we have obtained a pure culture.*

It is evident that by means of this method we are also able to introduce a single cell directly into the flask with nutritive solution, or the individual cell is allowed to develop in a drop suspended in a moist chamber, and the growth which forms is then transferred to the flask.

It was by this method that HANSEN prepared all his earliest pure cultures, with which he carried out his fundamental researches on alcoholic ferments.

Solid nutrient media have also been employed for the preparation of pure cultures for use in physiological investigations. The foundation of such methods was laid by SCHROETER (1872), who, in his researches on pigment-bacteria, employed slices of

potato among other nutrients. He had observed that when such slices had been exposed for some time to the air, specks or drops of different form and colour made their appearance. Each of these specks contained most frequently *one* species of micro-organism.

KOCH considerably improved this method. He at first prepared his pure cultures by means of streak growths in nutritive gelatine. Afterwards he devised a far better method, the so-called plate-culture method (1883). He proceeds in the following manner. A trace of the crude culture is transferred to a large proportion of sterilised water. From this a small quantity is transferred to a flask containing, for instance, a mixture of meat-broth and gelatine warmed to 30° C. The flask is shaken in order to distribute the germs, and the contents poured on to a large glass plate, which is then covered with a bell-glass. The gelatine quickly sets and the germs remain enclosed in the solid mass. In a few days they develop to colonies—points or specks which are visible to the naked eye. The purity of the specks of bacteria in the gelatine is ascertained, according to KOCH, partly by their appearance, colour, form, etc.

When regarded more closely it will be seen, however, that there is no *essential* difference between this distribution of the germs in the liquid gelatine, and the former dilution by means of liquids. The same uncertainty is always present: neither the macroscopical observation of the appearance of the colony nor the microscopical examination of its contents gives any surety of its only containing one species.

The only possibility of securing a really pure culture in the gelatine consists in the direct observation of one individual germ and its development.

HANSEN has done this in the case of yeast-cells, and the method which he contrived for the purpose is as follows. *The layer of gelatine formed by the solidified nutritive liquid is arranged in such a way that the position of the isolated germs can be observed under the microscope.* The position of these germs, then, is accurately marked, and the cell can be seen to develop and propagate step by step.

For the glass-plate is substituted a round cover-glass of

about 30 mm. diameter. This is fastened to a glass-ring, which again is cemented to a thicker glass, thus forming the moist chamber previously described (Fig. 2), which is adapted to the purpose, and carries a layer of solid gelatine on the inside of its upper surface. The essential point in HANSEN'S method is, that the leading principle—"the starting-point of a pure culture must be a single cell"—is consistently carried out, which is not the case in KOCH'S method. The germs must be so sparsely distributed that comparatively few are present in the gelatine layer; the chamber is then either allowed to remain under the microscope, in order that the multiplication of the germs may be directly followed, or the positions of well isolated germs are marked, either by dividing the glass-cover into small squares or by means of the object marker,

FIG. 9.—Moist chamber with edged squares.

and the apparatus is placed in the incubator, or at the temperature of the room, until the colonies are completely grown. In the author's laboratory moist chambers like that represented in Fig. 9 are used, the cover-glass being etched with 16 squares and figures. The situation of the cells is then marked on a sketch plan which shows all the etched figures and squares. On one cover-glass there may be 50 to 60 well isolated germs. When the colonies are completely developed, they are transferred to flasks by means of a small piece of platinum or copper wire, which has been previously ignited and cooled. During this transference the culture is momentarily in the air, and is here exposed to contamination. But the danger of contamination at this, the single weak point, is reduced to an insignificant minimum, and disappears if the operation is performed in a

small enclosed germ-free space; as, for instance, in a small chamber with glass sides which is sufficiently large to admit the apparatus and the hands of the experimenter. In this way the transference of the colonies is effected with all possible security. From the first flask the culture can be transferred without contamination to a continually increasing number of larger flasks. Thus, HANSEN'S method approaches the desired end as nearly as is possible, and is consequently employed everywhere in exact experiments of this kind.

As early as 1883, KOCH'S method of plate-culture was tested by HANSEN. He prepared a mixture of two species of yeast which can be distinguished from each other microscopically, namely, Saccharomyces apiculatus and a species of the group Sacch. cerevisiæ. This mixture was introduced into wort-gelatine, and after shaking was poured onto a glass-plate. Of the specks formed, about one half contained one species exclusively, the other half the other species, and in one of the specks both species were found.

A similar control was carried out for the bacteria by MIQUEL (1888), who introduced 100 colonies from a plate-culture obtained in an air-analysis into 100 flasks containing meat-broth with peptone. The examination of the growths developed in the flasks showed that they contained 134 different species of micro-organisms. This evidently depends upon the fact that it is very difficult, and often quite impossible, to separate all germs of bacteria and other organisms from each other by simply shaking the gelatine mixture. This test proves therefore that the plate-culture involves very material errors.

HOLM has subjected the method to a thorough analysis (1891), and has experimented with a considerable number of yeast-species, absolutely pure cultures of which were prepared by the above-mentioned method of HANSEN'S. The result of 23 series of experiments with different mixtures was, that only in a single case were 100 colonies developed from 100 cells; that is to say, all the colonies were pure cultures. In all the other series the method proved faulty. In the most unfavourable case 100 colonies were yielded by 135 cells, and the average number obtained

was 100 colonies from 108 cells. This proves the plate method to be faulty also in the case of yeast.

Thus, the advantage of HANSEN'S method over KOCH'S for the pure cultivation of yeast is, that it has a certain starting-point. Even if the plate cultures are repeated several times, one can never be certain whether the desired result has been attained or not. With regard to the bacteria, however, it is generally impossible to secure a starting-point from one individual cell. In such cases KOCH'S plate culture is still the best method we have.

8. *COUNTING THE YEAST-CELLS.*

In the yeast and spirit manufactures it is of importance to determine the *multiplying capacity of the yeast-cells* during the growth of the yeast. This must naturally be

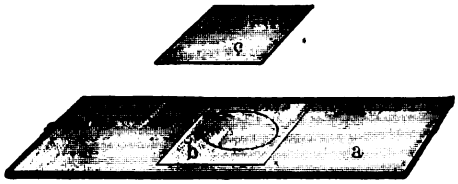

FIG. 10.—Hæmatimeter: *a*, object-glass; *b*, cemented cover-glass with circular opening; *c*, cover-glass.

effected by a direct counting of the number of cells which occur in a determinate volume of the liquid at different stages of the fermentation. Experiments having this object in view have been undertaken especially by DELBRUECK, DURST, HANSEN, HAYDUCK, and PEDERSEN, whilst FITZ has applied the method of counting to bacteria.

The counting is performed by means of an apparatus constructed by HAYEM and NACHET (Fig. 10), which was first employed for counting the corpuscles of blood (hence termed *hæmatimeter*). The late Prof. PANUM, of Copenhagen, was the first to employ this apparatus for counting micro-organisms, in order to determine their multiplying capacity. The hæmatimeter consists, as shown in Fig. 10, of an object-glass on which a cover-glass of *known thickness* (0·2 mm.,

for instance) is cemented, and from the centre of which a disc has been cut out. A small drop of the liquid containing the cells is brought into the cavity thus formed, a second cover-glass is placed over the opening, and thus rests on the cemented and perforated cover-glass. The drop of liquid must not be so large that the pressure of the cover-glass causes it to flow out from the enclosed space, yet it must be high enough to be in contact with the cover-glass. The thickness of the layer of liquid is then known. In order to determine the other two dimensions, and thus be able to work with a given *volume* of liquid, one of the well known forms of micrometer, *e.g.*, a thin piece of glass on which 16 small squares are engraved, is introduced into the eye-piece of the microscope. The actual value of each of these squares is known when a given system of lenses is employed, and thus, when the square is projected on the object, a small prism of known volume is defined. In certain cases it may be more expedient to make use of an appliance constructed by ZEISS, of Jena, from the instructions of THOMA, which consists of a fine system of squares of known size, engraved on the object-glass itself at the bottom of the cavity. This also improves the microscopical definition of the cells which are on the bottom of the chamber.

When it is merely desired to determine the rapidity with which the cells multiply, or to make repeated observations of the number of cells in the *same* volume, it is quite superfluous to determine its size; it is simply necessary to work always with the same volume.

It is always necessary that the sample taken should be a *fair average one.* In most cases it must be diluted and thoroughly agitated for a long time, in order to obtain an equal distribution of the cells; the specific gravity of the liquid must also be such that it will allow the cells to remain suspended in it for a short time. A small drop is then withdrawn in a capillary tube, transferred to the counting apparatus, and covered with the cover-glass. The apparatus is allowed to remain at rest for some time, in order that the cells may settle to the bottom of the enclosed space, and on this account the specific gravity of the liquid must not be greater than will allow this to take place in a convenient time. Both these

requirements are generally satisfied by the wort employed in breweries.

If it is found that the determinate volume contains too many cells to be counted with certainty, the liquid must be diluted. This may be advisable for other reasons, partly to prevent the formation of froth, which may otherwise form abundantly from the violent agitation, and partly to isolate the single cells which frequently cluster as colonies in the wort, and are not always separated by shaking, and, finally, in order to bring about a discontinuation of the fermentation and multiplication of the yeast-cells at the beginning of the experiment.

HANSEN found that dilute sulphuric acid (1 : 10) on the whole answers these requirements; hydrochloric acid, ammonia and caustic soda may also be used, but they are not so good. If very great dilution is required, distilled water may be added, after the addition of one to two volumes of dilute sulphuric acid.

When the different volumes of liquid are measured with accuracy, and particular care taken that the cells are thoroughly distributed by vigorous and prolonged shaking, the determination can be made with great accuracy. Two similar dilutions must always be made, and samples taken from each for counting. As a matter of course, experiments must also be made to determine the number of the small squares whose cell contents must be counted in order to arrive at a true average. Such a counting and determination of the average numbers is continued until the number finally obtained is found to have no further influence on the average value. The number of countings necessary, and the accuracy generally, depend on the experience and care of the observer. HANSEN found that, as a general rule, it was sufficient to count the cells in 48 to 64 small squares.

CHAPTER II.

EXAMINATION OF AIR AND WATER.

WHILST chemical investigations into the composition of air and water reach back into remote times, these researches have only been attempted during the last few decades from a biological point of view. Yet such ample materials have already been accumulated as to justify the assertion that the microbiological composition of both air and water is apt to play an important part, not only in relation to hygiene, but also zymologically. This is obvious from the fact that the various germs which are capable of producing diseases in fermenting liquids, *can live in air and in water.*

This part of zymology is, however, still in its infancy, and it may be justifiably asserted that rather too great importance has been ascribed to air, and, still more, to water in their zymological bearings. In the case of normal manufacturing conditions, *i.e.* where work is carried on with due insight into the principles of rational fermentation, neither of the two factors in question—*i.e.* the microbiological composition of water and air—generally affects the operations in a *direct* manner; but the case is quite different if that insight is wanting, it then becomes possible for those micro-organisms of water and air which are capable of developing under the circumstances, to take root and propagate. But then it is neither water nor air that are essentially to blame.

The great significance of the biological analysis of air and water lies in the valuable hints which it can give with regard

to the dangers that may arise from the neglect of the requisite considerations alluded to above.

The discoveries of SPALLANZANI (mentioned in the last chapter), and of later investigators on the *generatio spontanea* first gave rise to the study of micro-organisms of the air. PASTEUR especially showed that these micro-organisms are of vital importance to the fermentation industries, when he proved that the air contained both bacteria and alcoholic ferments.

It will be of interest to glance at the analytical methods by which the germ-contents of the air have been determined.

The majority of the **analyses of air** have been undertaken with the view of obtaining some light on the obscurity which envelops most contagious diseases, nearly all of which are, as is well-known, attributed to the agency of micro-organisms. With regard to the organisms of fermentation, these have been investigated chiefly by PASTEUR, and later by HANSEN. The French *savant* stated that these germs are always floating about in the air, but that *they are present in much larger quantities in the dust which settles on the vessels and apparatus employed.* The true alcoholic ferments are present in comparatively small numbers in the air, whilst the germs of mould-fungi are more frequent; he further showed, as was subsequently done by TYNDALL, that the germ-contents of the air vary both with regard to quantity and the species present. These results were obtained by exposing beer-wort, wine-must, or yeast-water containing sugar, in open, shallow dishes, at different places, and examining their contents after some time for microscopical germs. PASTEUR also employed for this purpose the so-called vacuum-flasks, containing nutritive liquids and rarefied air. On opening the flask a sample of germ-laden air could be drawn in.

The most important air-analyses undertaken in recent years are, without doubt, those carried out by MIQUEL, the director of the laboratory specially arranged for this purpose at Montsouris, near Paris. His fellow-worker, FREUDENREICH, has also made valuable contributions to our knowledge of this subject.

MIQUEL performed his first experiments with a so-called

aëroscope (Fig. 11), which is constructed in the following manner:—A bell-shaped vessel, A, is provided with a tube, C, through which air can be aspirated. A hollow cone, shown in the left-hand figure, is screwed into the bottom of A; the mouth

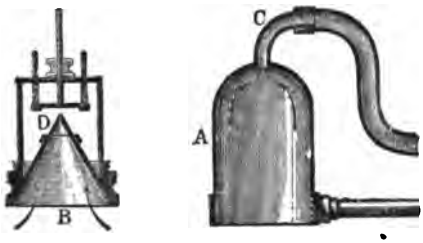

FIG. 11.—Aëroscope.

of the cone, B, points downwards; in the apex, D, of this cone there is a very fine opening through which the air is aspirated, and immediately over this opening is fixed a thin glass-plate covered with a mixture of glycerine and glucose. The particles carried in by the air settle to a great extent on the viscous mixture. The intercepted micro-organisms are distributed as equally as possible on the glass-plate, and counted under the microscope. This method is so far defective in that it gives no information on the most important point, namely, which and how many of the intercepted germs are actually capable of development.

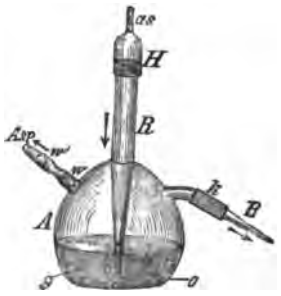

FIG. 12.—MIQUEL'S Apparatus for air-analysis.

In order to determine the number of germs capable of development, and also their nature, MIQUEL employs the following apparatus (Fig. 12). The flask *A* has fused into it a tube, *R*, tapering below and nearly reaching to the bottom; the upper end of this is fitted with a ground cap, *H*, provided with a narrow filter-tube containing sterilised cotton-wool, asbestos, or glass-wool, *as*. On one side of the flask is a tube, *Asp*, which is constricted in the middle and is provided with two cotton-wool plugs, *w'* and *w*. On the other

side is another glass tube, which is connected by rubber, *k*, with the tube *B*, which is drawn out to a point, and closed by fusing the end. The flask is filled with distilled water, and the whole apparatus sterilised. When the apparatus is to be employed, the tube *Asp* is connected with an aspirator; for instance, a bottle filled with water and provided with an outlet cock; the cap *H* is taken off, and the air then passes, bubble by bubble, through the opening *o*, through the water *g*, and out through the cotton-wool plugs of the tube *Asp*. Since all the germs of the air are not retained by the water when the air-bubbles ascend through the latter, the cotton-wool plug *w* is intended to catch those which get past the water. When the experiment is finished, the cap *H* is replaced over the tube *R*. By blowing through *Asp*, the liquid is made to ascend in *R*, in order that any germs which may have settled on the walls of the tube may be washed down into the liquid. Then, by blowing with greater force, the inner cotton-wool plug *w* is driven down into the liquid, and its germs shaken off into the latter. After sterilising the thin tube *B* in a flame, the point is nipped off, and the liquid is now—by blowing through *Asp*—transferred, drop by drop, into a large number of flasks containing sterilised broth.

The main point here is, by means of preparatory experiments, to obtain such a dilution of the air-infected water that a considerable proportion of the small flasks (one-half for example) remain sterile after inoculation; or several samples of the water may be diluted to different degrees, and a series of flasks inoculated from each dilution. When a large number of the flasks do not show any development of organisms, there is a *certain probability that in each of the remaining flasks in which growths have developed, only one germ has been sown.* A simple calculation will then show how many germs capable of development in the medium employed were present in the volume of air aspirated through the original flask ("fractional cultivation").

By these methods of investigation MIQUEL found that similar volumes of air in the same locality contained at different times a different number of bacteria. A prolonged shower of rain purifies the air from bacteria to a marked

extent, and their number continually diminishes as long as the earth is moist; but when the ground dries, it gradually increases again. Thus in the dry seasons of the year the number of bacteria is usually the greatest, whilst the mould-fungi, which thrive best in moisture, and whose organs of reproduction project upwards, are most abundant in the air during the wet seasons. The purest air is found in the winter time; the air of towns is less pure than that of the country; germ-free, or nearly germ-free air is found at sea and on high mountains. In certain localities—hospitals, for instance—the air has been found to be very rich in bacteria; in one case even 50 times richer than the air in the garden at Montsouris.

An entirely different method for the examination of the organisms contained in air is that employed in KOCH'S laboratory, and more completely developed by HESSE. A glass tube, about 1 meter long and 4 to 5 cm. wide, is closed at one end with a perforated india-rubber membrane, over which another non-perforated cap is bound. A little liquid nutritive gelatine is then poured into the tube, after which the other end of the tube is closed with an india-rubber stopper, through which passes a glass tube plugged with cotton-wool. The whole apparatus is then heated sufficiently to render it sterile, after which the tube is placed in a horizontal position, so that the gelatine sets in a layer in the lower part of the tube. When the air is to be examined, the outer india-rubber cap is removed, and air slowly drawn through the tube. The germs contained in the air settle down on the gelatine, and after the aspiration is concluded the tube is again closed and placed in the incubator, where some of the germs produce visible colonies, which are easily counted. The results show that with a sufficiently slow current of air, the bacteria, which are often floating about in the air in larger or smaller aggregations, frequently clinging to dust-particles, settle sooner than the mould-spores; so that the gelatine in the front part of the tube generally contains the majority of the bacteria colonies, whilst the mould-spores develop further along.

HUEPPE, v. SCHLEN, and others, employ liquid gelatine for air analyses, the air being aspirated through the gelatine, after which the latter is poured onto glass-plates.

FRANKLAND, MIQUEL, and PETRI, use porous solid substances for the filtration of air for analytical purposes; as, for example, powdered glass, glass-wool, sand, sugar, etc. The sand-filter employed by PETRI is 3 cm. long and 1·8 cm. wide. It is filled with sand, previously ignited, the size of the grains being from 0·25 to 0·5 mm. Two such sand-filters are placed one behind the other in a glass-tube. The first filter should retain all the dust-particles containing germs, whilst the other filter should remain sterile, and thus serves as a control. The sand charged with germs is distributed in shallow glass dishes and covered with liquid gelatine. The germs contained in the dust-particles will then develop colonies in the gelatine.

When samples of air are to be sent from one place to another, these air-filters will answer the purpose. On receipt of a sample, the sand may be washed into gelatine or, preferably, into sterilised water. After vigorously agitating the water, it is added in drops to flasks containing nutritive liquid, or it may be used in plate-cultures.

MIQUEL has raised an objection to the employment of gelatine plates for this purpose, based upon numerous experiments. He asserts that many bacteria, when exposed to a temperature of 20° to 22° C., require a fortnight's incubation before developing distinct colonies in gelatine; on the other hand, there are species which will very soon liquefy the gelatine, thus rendering further observation impossible. The same is the case with the mould-fungi, which will often spread over the plate in a few days. Thus, it becomes necessary to count the colonies at an early stage when many are not yet visible. An additional drawback to the gelatine plates is, that the development cannot take place at a temperature higher than 23° to 24° C., otherwise the gelatine will become liquid; but many species of bacteria give a fair development only at considerably higher temperatures. Other species, moreover, do not develop in gelatine at all, but only in liquids. Finally, it is urged as a very material objection to the gelatine-plates, that many of the colonies consist of several species. MIQUEL proved this by introducing the colonies, one by one, into meat decoction with peptone, and then again preparing plates from these growths. This is in part due to the fact

that bacteria, as shown by PETRI, often occur in aggregates in the air, and these will either fall directly onto the gelatine-plate or become mixed in the liquid gelatine, where it will be very difficult to separate the individuals from each other by agitation.

HANSEN'S investigations of the air were made from 1878 to 1882. The main object of his investigations was to throw light on questions affecting the fermentation industries. As is known, his researches on Saccharomyces apiculatus (1880) were partly based on work of this nature. Since the question concerned the organisms which occur in brewing operations, the choice of a nutritive liquid was easily determined, namely, wort as ordinarily employed in breweries. The apparatus employed consisted either of ordinary boiling flasks closed with several layers of sterilised filter-paper, the contents of which were boiled for a certain time, or of vessels similar to Pasteur's vacuum flasks, the necks of which were drawn out to a fine point, and closed with sealing-wax while the contents were boiling. A little below the point a scratch was made with a file, so that the point might be easily broken off when it was desired to admit air.

When these flasks had been filled with the air of the locality to be examined, they were again closed with sealing-wax and thoroughly shaken in order to mix the contents of the infiltrated air with the liquid. The flasks were then put aside for a longer or shorter time, lasting in some cases for six weeks, and their contents examined under the microscope.

In these investigations HANSEN often found that the wort remained bright and apparently unchanged, even although a growth had taken place. Hence, the examination with the naked eye alone cannot be relied on. He names the following forms which, when present in a feeble state of growth, cannot be detected macroscopically :—Aspergillus, Mucor, Penicillium, Cladosporium, Bacterium aceti and Pasteurianum, and Mycoderma cerevisiæ. Even when these micro-organisms have formed vigorous growths, the wort used has remained bright.

It was further shown that pure cultures may often be obtained by the use of these flasks, when only one species was drawn into the flask with the air. It very seldom happened

that three or four species were found in the same flask. This arises from the fact that only a very small volume of air enters each flask. The advantages of this are evident:—a true knowledge of these germs can only be obtained when they have developed; in cases where several germs penetrate into the same flask, the strongest germ would by its growth, in all probability, prevent the development of the others, so that these would not be detected in a subsequent examination. At the same time this method necessitates the opening of a large number of flasks, which makes the operation cumbersome and costly. As the flasks only show what was present in the air at the moment of opening, Erlenmeyer flasks were also used to give supplementary information, for which purpose they were allowed to remain in the same locality for some length of time, in some cases as long as 48 hours.

After these preliminary remarks we will give a brief summary of the results obtained by HANSEN.

He confirmed the statement first made by PASTEUR, that the air *at adjacent places, and at the same time, may contain different numbers and different varieties of organisms*; and he found that this holds good for adjacent parts of one garden. HANSEN states, in discussing the distribution of micro-organisms, that those forms, for instance, which in the first half of July commonly occurred under the cherry trees in the garden, were in the latter half of the same month entirely absent from their locality; further, that organisms which at one time were found under the cherry trees, but not under the vines, were to be found later only under the latter. As a proof of the inequality of distribution of the organisms, he shows that flasks opened in the same place in the same series of experiments often had the most diverse contents.

The experiments with vacuum flasks have further taught us that the micro-organisms of the air often occur in *groups* or *clouds*, with intermediate spaces, which are either germ-free or only contain a few isolated germs. As the organisms are not generated in the air, but on the earth, it follows that their presence in the air must be dependent on the condition of the surface of the ground, which again depends, in certain respects, on the weather.

HANSEN'S numerous analyses have further proved that the *Saccharomycetes occur comparatively seldom in the dust particles of the air.* Their number in the open air increases from June to August to such an extent that flasks at the end of August and the beginning of September are frequently infected with these organisms, after which a decrease takes place. The Saccharomycetes which are found at other times of the year in the atmosphere, may be regarded as unimportant in numbers and accidental in occurrence. As most species of the Saccharomycetes have in all probability—like Saccharomyces apiculatus—their winter quarters in the earth and their places of growth on *sweet succulent fruits,* these latter must be considered as the most important source of contamination. During the same season *bacteria* are also found in the largest numbers. This constitutes a real danger in technical operations, since the wort, when spread in a thin layer on the open coolers, is exposed to a great source of contamination from the atmospheric germs.

Bacteria are found in the flasks in somewhat greater number than the Saccharomycetes, whilst the *mould-fungi occur in still greater numbers.* Amongst the latter Cladosporium and Dematium are especially prevalent in gardens, and after these Penicillium; whilst Botrytis, Mucor, and Oidium occur less frequently.

After HANSEN has thus stated which of the micro-organisms existing in *the open air* are capable of developing in flasks with sterilised wort, he proceeds to communicate the results of his examination of different parts of the brewery.

When *grains* (draff) are allowed to stand in the open air, they evolve acid vapours, and since they always exhibit a rich growth of bacteria when they remain exposed for a short time, it is important to study the conditions of the air in the neighbourhood of the heaps of grain. It was found that only 30 per cent. of the flasks opened in these areas became contaminated, and of these 3·6 per cent. with Saccharomycetes and 2·4 per cent. with bacteria, whilst parallel experiments in the garden gave a contamination of about 44 per cent., of which 8·5 per cent. were bacteria. The air near the grains thus contained fewer bacteria than the air of the garden. The most

abundant contamination here was that of mould-fungi, as in all other localities. After a thorough examination HANSEN came to the conclusion that, without doubt, *scarcely a single organism which entered the flasks proceeded from the grains.* At all events the great abundance of bacteria in the grains does not bear any relation to the above-stated result, which tends to show that in this, as in other cases, air does not take up organisms from moist surfaces.

This, however, must not be misunderstood to mean that grains may be allowed to accumulate, without risk, and that after removal, the residue may be exposed to the weather. It is clear that this would constitute a great danger. When the residue becomes dry and is blown about in the air as dust, masses of bacterial germs will be carried up at the same time, and will, without doubt, constitute a source of constant bacterial contamination. For this reason, places where grains have remained for any length of time must be washed with lime-water or, preferably, with chloride of lime.[1]

In a corridor which led to a room where the *barley was turned,* the flasks always received a greater contamination than anywhere else; *bacteria* especially were found in great abundance.

On the *malt floors* the condition of the air was also characteristic; it always contained a very strong *growth of mould.* In the case in question this growth consisted of Eurotium Aspergillus, which was othewise rare. On the malt itself, as always, Penicillium glaucum occurred the most frequently.

The greatest interest, however, attaches to his examination of the different *fermenting-rooms,* partly in "Old Carlsberg" brewery and partly in the brewery "N." In the former the air contained fewer organisms than in any of the localities examined during the whole research; in the fermenting-cellars of the brewery "N," on the contrary, a large number of flasks (55·75 to 100 per cent.) were infected. The

[1]The germs are not killed during the treatment of the grain in *drying machines.* Such apparatus, therefore, constitute a very great danger in the brewery, in cases where dust-clouds of bacteria can be transported from the dried grains to the open coolers or into the fermentation-vessels.

organisms which occurred in the air of these cellars were: Saccharomyces cerevisiæ, Mycoderma cerevisiæ, Sacch. Pastorianus, Sacch. ellipsoideus, Torula Pasteur, and other yeast-like forms; further, Penicillium, Dematium, Cladosporium, and rod bacteria. HANSEN was thus enabled, by a favourable chance, to sharply contrast the state of the air in the most important part of these two breweries: on the one hand an almost germ-free air, on the other hand an atmosphere teeming with germs. That the product of the latter place must have been influenced by the atmospheric conditions then existing admits of no doubt; it serves to impress us with one of the most important of all facts connected with the practice of the fermentation industries. *The air in the fermenting room itself may contain a multitude of those germs which are productive of the most calamitous results;* it is, however, possible to keep the air free from these invisible germs, and it admits of no doubt that, on the one hand, the purification of the air entering the fermenting-room by passing it over brine, and, on the other hand, the very rigidly maintained order and cleanliness in the cellars of the Old Carlsberg brewery stand in direct relation to the above-mentioned result. HANSEN'S investigations, therefore, point a moral which cannot be too frequently emphasised.

Zymotechnical examinations of **water** according to the principles laid down by HANSEN (a description of which is given in the sequel) were carried out on a large scale by J. CHR. HOLM (Carlsberg Laboratory), and also in the author's laboratory.

HOLM'S researches show that among the various micro-organisms the *moulds* are those which develop most quickly in flasks containing wort and beer, and generally also those which occur in largest numbers in the flasks. *Penicillium glaucum* and *Mucor stolonifer* were found among them.

Next to the moulds come the *bacteria*, if wort is infected with the water, whilst if sterilised beer is used, they developed only scantily. The following bacterial forms were found:— *Bacterium aceti, Bact. Pasteurianum*, a third form which made the beer slimy and ropy; and lastly, species were frequently found which imparted a disgusting smell to the wort.

Of rarest occurrence in the experiments here referred to

were the *yeast-like cells.* HOLM did not find any growth of *Saccharomycetes*; yet some Torula forms and *Mycoderma* occurred.

The number of these germs varied according to the different times of the year, yet did not seem to be dependent on the seasons,—the rain-fall, etc., and the condition of the surface water had great influence. As being of practical importance we may mention the discovery of strong contamination injurious to wort and beer, in reservoirs situated near granaries and malt-lofts and not sufficiently protected against the dust. It was also shown that water which had been filtered through charcoal filters contained much larger numbers of wort bacteria than the unfiltered water.

The water analyses made in the author's laboratory during a period of more than ten years have given the following chief results:—The water samples in very few cases were found to contain *Saccharomycetes* (culture-yeasts or wild yeasts). In one series of analyses species of *Sacch. anomalus* and *S. membranaefaciens* were met with. The bacteria noted by HOLM which produced slime-formations or imparted a *putrid smell* to the wort, occurred very frequently. If a pure yeast was infected with such species and used for pitching hopped wort, these bacteria did not usually develop further. Although, however, the bacteria did not develop during the fermentation, a difference was often observable between the condition of this beer and that of the beer fermented with pure yeast. *Acetic acid bacteria* were not infrequently found in the analyses, and were usually able to assert themselves in the flasks, even in competition with rival species. In a few cases the experiments with wort showed a growth, and sometimes even an abundant one, of *Sarcina* forms, which did not occur in the parallel series with sterile beer. They rendered the wort turbid and imparted a peculiar smell to it. Among the moulds the following were the most frequent:—*Penicillium, Aspergillus, Mucor stolonifer, M. Mucedo, Oïdium lactis* and *Dematium-like* forms. In the water conduits of the breweries a coherent layer of *Crenothrix* was often found.

It has been proved in many cases that water received a very considerable contingent of its wort and beer organisms in the

reservoirs or in the conduits, and it may safely be asserted as the result of many years' experience, that brewery water receives its most dangerous contaminations in the brewery itself.

Biological analyses of *natural* and *artificial ice* have shown that in both sorts of ice organisms can exist capable of developing in wort and beer. Sarcina-like bacteria can also be introduced along with ice into these liquids and may develop freely in them.

If large quantities of water are to be analysed, it is of the utmost importance to take due care that *real average samples* are obtained.

HANSEN *gave the following method for the zymotechnical analysis of air and water, a method based upon a long series of comparative trials.*

The principle of this method of air and water analysis is as follows:—For brewing purposes it is only necessary to know *whether the water and the air contain germs capable of developing in wort and beer.* This cannot, as was formerly assumed, be ascertained by means of the meat decoction peptone gelatine employed in hygienic air and water analysis. The zymotechnologist has this great advantage over the hygienist, that he is in a position to *make direct experiments* with the same kind of liquid as that employed in practice, namely wort. *All disease germs that have hitherto been shown with certainty to occur in beer are also capable of developing in wort.* HANSEN'S *comparative investigations have proved beyond dispute that the use of gelatines introduces great sources of error.* Thus, for instance, in a series of comparative experiments with corresponding samples of water, the following numbers were obtained:—In KOCH'S nutritive gelatine: 100, 222, 1000, 750, and 1500 growths were obtained from 1 c.cm. of water; in wort 0, 0, 6·6, 3, and 9 growths; whereas, in beer, none of these water-samples gave any growth. In another series, KOCH'S gelatine gave for 1 c.cm. of water 222 growths, wort-gelatine 30; but none of the flasks containing wort or beer, after infection with the water, showed any development of organisms. Thus, only very few of the great number of germs living in the water developed in wort or beer.

HANSEN has further shown, that in zymotechnical analyses of water and air, it is a mistake to employ gelatine at the outset, and then to transfer the colonies that have been formed into wort. Thus, he demonstrated by experiments that several of the bacterial germs existing in atmospheric dust and in water are capable of developing in nutritive gelatine, but not in wort; but several of these species become invigorated to such a degree after having formed a new growth in the gelatine, that they are then enabled to develop in the less favourable medium, wort. In such cases the experimenter is therefore deceived. Another, and a still greater, objection to the gelatine method is, that several important organisms *do not develop at all* when transferred directly to the gelatine in the enfeebled condition in which they generally occur in atmospheric dust and in water.

Based upon these observations, HANSEN devised the following method: Small quantities of the water, either in its original state or diluted, are added to a series of *Freudenreich* flasks containing either sterilised wort or beer.[1] After incubation at 25° C. for fourteen days the contents of the culture-flasks are submitted to examination. If only a part of them show any development, the rest remaining sterile, it may be assumed with approximate certainty that each of the flasks belonging to the former set has received only *one germ*. Information is thus gained concerning the number of germs capable of development existing in a determinate volume, and the different germs are also under more favourable conditions for their free development. An exact examination will show to what species these germs belong.

Although, in this method, the wort-cultures give a very small number of growths in comparison to the plate-cultures, yet in many cases the *number of wort-growths will still be too high*, for these growths are able to develop in the flasks undisturbed and without hindrance from other organisms, but when wort is mixed with good culture-yeast in the fermenting vessel, many of these germs will be checked. Further, the flasks which show a formation of mould will have no impor-

[1] In the analyses of air the germs are aspirated into water, or first into cotton-wool and then transferred to water.

tance for the brewery, but only for the malt-house. By way of a closer approximation to practical requirements, HANSEN proposes the following method of procedure. *The flasks containing a development of yeasts and bacteria* are divided into two groups: (1) those in which the growths soon appeared, and (2) the remainder, in which they made their appearance later; as, for instance, after five days. Among the latter growths are those species which develop less readily in wort; and in the brewery these will therefore be generally suppressed by the yeast, and are consequently of less importance in the examination of water or air. Analyses according to this method have been executed by HOLM, WICHMANN, and several others, and the method has always been used in the author's laboratory.

For the control of air and water filters KOCH'S gelatine method can be advantageously used.

CHAPTER III.

BACTERIA.

THE more our knowledge of these micro-organisms is extended, the more difficult it is to give a general definition of them. They are known in all forms, from the smallest specks or spheres to green, alga-like filaments; and they occur in nearly all possible localities, under the most varied conditions. According to their action a distinction is made between *pathogenic*, *zymogenic*, and *chromogenic bacteria*, or such as produce disease, fermentation, or coloration respectively.

The first knowledge of these forms was obtained by placing small quantities of the different substances under the microscope and examining them with high powers. In putrefying meat very small spherical bodies were found, which clearly multiplied by successive divisions; in sour milk short, rod-like bodies occurred; and in putrefying vegetable matter large spherical bodies and long, fine, thread-like forms; in saliva, on the contrary, very fine, spirally-twisted threads were found. On this account it was convenient to provisionally retain these *forms*, and to describe them as so many distinct *species*. COHN especially has earned credit in this respect, since to him is due the first systematic classification of bacteria.

We will first consider the various forms and individuals somewhat more closely. As stated above, the bacteria in their simplest form occur as spherical bodies of different sizes, often so small that they can only just be seen even with the strongest powers, and only give evidence of their existence

as organisms during propagation by division. They are accordingly divided into *Macrococci* and *Micrococci* (Fig. 13 *a*). When the spheres occur in pairs, they are called *Diplococci* (*b*); they also appear in groups consisting of four individuals, *Sarcina* (*b*); or of a greater number, arranged irregularly, or in chains, *Streptococci* (*c*). From the coccus forms there is a gradual transition to the rod forms—*Bacterium*, *Bacillus* (*e*), which vary greatly both in length and thickness. When the rods are

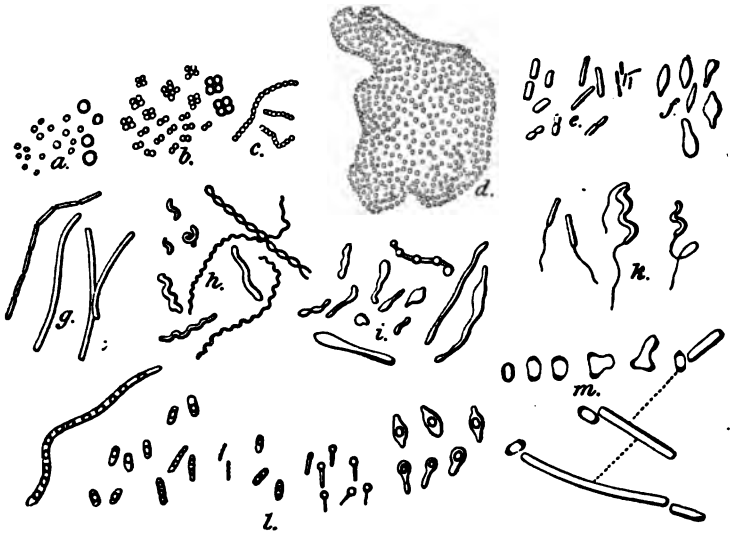

FIG. 13.—Growth-forms of Bacteria (in part schematic): *a*, Cocci; *b*, Diplococci and Sarcina; *c*, Streptococci; *d*, Zoogloea; *e*, Bacteria and Bacilli; *f*, Clostridium; *g*, Pseudo-filament, Leptothrix, Cladothrix; *h*, Vibrio, Spirillum, Spirochæte, and Spirulina; *i*, Involution-forms; *k*, Bacilli and Spirilla with cilia or flagella; *l*, Spore-forming Bacteria; *m*, Germination of the Spore (Bacillus subtilis).

enlarged in the middle and taper towards the ends, *i.e.*, spindle-shaped, we have the *Clostridium* form (*f*). If the rods are elongated so as to become more or less thread-like, they are called *Leptothrix* (*g*), which may also occur as *pseudo-filaments* (*g*), when several rods are arranged lengthwise, or as *Cladothrix*, when they lie so close to one another and in such a way that they become seemingly ramified. Rods and filaments frequently assume wavy or spiral forms (*h*); when they are only slightly curved, we have the *Vibrio* form; when the spirals are more prominent, the *Spirillum*, and *Spirochæte*

forms; when they intertwine like a plait of hair, the form called *Spirulina* is produced. To these must be added the remarkable irregular, swollen, or curved forms which many bacteria can assume—*involution forms* (*i*); these are often brought into existence by unfavourable conditions of life, but in the case of certain species (acetic acid bacteria) form part of the regular course of development.

We will now select one of these forms and submit it to a thorough examination with a magnifying power of about 1000 diameters. Like every other cell, it contains protoplasm, a homogeneous, feebly refractive mass, in which infinitesimal particles can be detected here and there, especially if the cell is not in its most active growth. Sometimes a bright spot is found in the middle of the cell, which, from analogy to the higher plants, is considered to be a sap-cavity or vacuole. In some bacteria certain solid substances have been detected, as, for instance, sulphur grains in bacteria which live in water containing sulphur; in some species the plasma can, under certain conditions, be coloured blue by iodine, which indicates the presence of substances resembling starch.

Surrounding this protoplasmic body we find a *cell-wall* or *membrane*. An examination of this by means of staining will generally show that its outer layers are swollen up into a gelatinous mass, which becomes especially distinct when masses of bacteria are aggregated together. From a chemical standpoint it must be provisionally assumed that this cell-wall is of a different nature in different species. In some it reminds us of the cellulose of the higher plants, whilst in others it appears rather to resemble the albuminoids in its properties.

Many bacteria contain blue, red, yellow, or green *colouring matter*, which sometimes causes very intense coloration. Under the microscope, however, the individual bacteria appear only very faintly coloured. It has not yet been determined with certainty in what part of the organism the colouring matter is situated. Some species of bacteria are *phosphorescent* under certain nutritive conditions.

A remarkable property of many bacteria is their *free locomotion*. This is either quick or slow, the bacteria rotating about their longitudinal axes, assuming the forms of open or

contracted spirals. In some of these motile forms we can observe, under high magnifying power, very fine cilia or flagella (Fig. 13*k*); whether these are to be considered as organs of locomotion is not determined; they are supposed to consist of plasma threads issuing from the interior of the bacterium, surrounded by a particular membrane.

The *propagation* of bacteria takes place by *division*. It has been observed in detail in the larger forms. The cells stretch themselves, fine transverse lines appear, which gradually increase in thickness and split into two leaflets; after this the organism separates into smaller rods which sometimes remain united, sometimes become detached (Fig. 13*g*). Long before a trace of these transverse walls can be observed, a staining of the organism will show that it consists of a series of segments, each of which corresponds to a subsequently-formed member. The newly-formed segment cells are all in the same plane. A division in two or three directions of space has been observed in certain micrococci (Pediococcus and Sarcina).

It was proved by careful investigation (especially by ZOPF), that *the same species of bacterium can occur in very different forms, e.g.*, as spirillum, leptothrix, bacillus, bacterium, and coccus; and we thus obtained the important addition to our knowledge of the history of these plants that the names quoted very often only express *growth forms* of the same species, and not distinct species. The following question, however, remains to be answered:—*Under what conditions does a species occur in this or that particular form?* Upon this point we know very little at present. It has been ascertained that both the temperature and the nature of the nutritive medium exercises considerable influence in this direction (comp. HANSEN'S observations with regard to acetic acid bacteria).

In the case of many bacteria formation of *spores* takes place in the following manner. The plasma in the cell becomes darker, and often distinctly granular; after that a small body appears, strongly refractive to light, which quickly increases in size, and is surrounded by a membrane; meanwhile by far the greater portion of the remaining plasma of the cell disappears, being used up in the formation of the spore; this is seen enclosed in a clear liquid which gradually disappears; finally

the cell-wall shrivels up, and only remains as a withered appendage to the ripe spore. In many cases the mother-cell swells up during the spore-formation. This organ is often termed a *resting spore* ("*Dauerspor*") for two reasons: first, because it actually possesses far greater durability and resistance to external influences than the vegetative rods [thus, in order to kill the spores of the hay-bacillus (B. subtilis) they must be boiled for three hours at 100° C., whilst the vegetative threads are killed at that temperature in twenty minutes]; and, secondly, because the spore formation generally takes place when the nutriment of the organism appears to be either exhausted or unfavourable to the further vegetative growth of the latter; the spores, then, serve to preserve the life of the organism during this critical period.

As soon as favourable conditions of nutriment and temperature again occur, the *spores germinate*. They first increase in size, and the contents lose their strong refractive power. A bacterium then grows out from the spore, the wall of which is sometimes seen to burst or divide into two shells (Figs. 13, 21). The full-grown rod then multiplies in the usual manner.

Bacteria are now frequently divided into *endosporic* and *arthrosporic* bacteria, of which the first-named form their spores in the interior of the vegetative rods, whilst in the latter group no such interior spore-formation has hitherto been observed; in these forms, members of a series of united generations of vegetative cells separate from the rest and assume the character of spores, immediately, without previous endogenous rejuvenescence, and originate new vegetative generations. A microscopically discernible difference between the arthrospores and the other members, however, occurs only in some cases, in that the walls of the latter spores grow thick (Cladothrix, Leptothrix, Bacterium Zopfii). Perhaps by continued investigation endogenous spores may also be found in all species of the arthrosporic group. It is only a supposition that the bacteria classified above must be considered as analogous to spores.

Finally, in the morphology of bacteria, we must mention the so-called *zooglœa* formation (Fig. 13 *d*). It is known that in all branches of the fermentation industries, in places where cleaning is not strictly attended to, slimy, fatty masses

may occur, which gradually increase in thickness. The cause of this is commonly a growth of bacteria occurring in such a manner that the single cells lie very close to each other, whilst at the same time the outer gelatinous layers of the cell-wall greatly swell up. During the continued growth of the bacteria the slimy layer increases in thickness, and often assumes certain characteristic forms. Such slimy masses —known in sugar manufacture as "frog-spawn"—occur both on solid and in liquid media. In a microscopical preparation, this bright, transparent mass of slime is not discernible. If specially treated—for instance, by immersing the preparation for some minutes in broth at about 35° C.—the slime-masses or capsules usually absorb colouring matter, and it then becomes possible to observe their form.

Many bacteria are very resistant to *low temperatures*. Thus J. FORSTER, B. FISCHER, MIQUEL, and others found that there are bacteria which multiply very well at 0°. Certain species withstand cooling down to 70° C., 110° C., even to 213° C., without being killed (FRISCH, PICTET, and YUNG). On the contrary, a number of bacteria capable of withstanding heat (*thermophilous*) have been discovered. The *bacillus thermophilus* described by MIQUEL multiplies actively even at 70° C., its growth stopping at 42° C. Other species do not develop at lower temperatures than 60° C. In excrements of animals several frequently occurring species were discovered, which continued growing even at 75° C., whilst at about 39° C. they ceased multiplying (L. RABINOWITSCH). To these thermophilous species belong several *lactic acid bacteria*, and probably also the various bacteria occurring in tobacco fermentation. It may also be mentioned that amongst the bacteria of this class a certain micrococcus was shown by F. COHN to be the cause of the spontaneous ignition of moist cotton.

The bacteria are very sensitive to the action of *light*, and many of them are quickly killed in direct sun-light. This fact plays an important part in the so-called "spontaneous purification" of rivers (H. BUCHNER).

Towards *mechanical concussion* bacteria and the other micro-organisms behave differently. MELTZER concluded, after

extensive experiments, that a slight concussion is beneficial to the life and propagation of micro-organisms; at a certain rate of vibration the propagation of the species is greatest; if the concussion is increased, the propagation is checked in proportion. But the optimum and maximum are different in different species.

PASTEUR made the important discovery that there are certain bacteria and other micro-organisms which do not require free oxygen, but are capable of producing very active decomposition of the fermenting material even when oxygen is excluded. He, therefore, distinguished two classes of micro-organisms, naming the last-mentioned *anaërobic* and the others *aërobic*. More recently DUCLAUX has stated that there are intermediate forms between the two extremes. As an example of anaërobic bacteria, PASTEUR'S bacterium of the butyric acid fermentation may be mentioned.

We will now pass in review the more important species which are of special interest in the fermentation industries.

1. *ACETIC ACID BACTERIA.*

The acetic acid bacteria were first thoroughly examined from a morphological and biological standpoint by HANSEN.

As early as 1837-38 the view was expressed by TURPIN and KUETZING that the acetic acid fermentation is caused by a micro-organism, which KUETZING described and delineated under the name of *Ulvina aceti*. Starting from this, PASTEUR, first in his treatise (1864) and subsequently in his work "Études sur le vinaigre" (1868), furnished experimental proof of the correctness of this view, and also gave a method, based on his results, for the manufacture of vinegar. He assumed that the acetic acid fermentation was caused by a single species of micro-organism which he called *Mycoderma aceti*. Subsequent research has, however, shown that there are different species of acetic acid bacteria. *As far as* PASTEUR'S *work was concerned, therefore, it was not at all a question of the employment of one definite, selected species.* His method consists in giving a large surface to the liquid employed—two parts of bright wine to one part of wine-vinegar—and then sowing on

the surface of the mixture a young film consisting of "mother of vinegar." When the temperature, the composition of the liquid, and all other conditions are favourable, the formation of acetic acid will proceed more quickly than in the older Orléans process. The installation is claimed to be cheaper, and the loss of alcohol not greater—at all events not to any appreciable extent—than in the last-named process. Yet, as far as the author has been able to learn, PASTEUR'S process is never employed. The uncertain results obtained may have been due to the fact that the composition of the nutritive liquid varies, and, especially, that the *bacterial culture was not a pure culture*, and might, therefore, also contain varieties of bacteria which possessed different properties, required different conditions for their growth, and, consequently, would give different products in varying quantities. This will hold good even in those cases in which the composite culture consists only of varieties which can produce vinegar. As early as 1879 HANSEN discovered that at least two distinct species are hidden under the name of *Mycoderma aceti*, which now go by the names of *Bacterium aceti* and *Bact. Pasteurianum*; and now a whole series of species are distinguished. To obtain the best results in this branch of industry it is likewise necessary to start with an absolutely pure culture of a methodically selected species. The old Orléans process still prevails in France. In this method the wine which is to be converted into vinegar is placed in casks, half-filled, at about 30° C., to which air has moderately free access. . The formation of acetic acid, as in Pasteur's process, takes place in consequence of the liquid being gradually covered with a film consisting of "mother of vinegar."—In other countries the German "quick vinegar process" is employed, in which the growth of bacteria, suspended in diluted spirit mixed with vinegar, is accelerated by coming into intimate contact with the air. This is brought about by allowing free access of air, by dividing the liquid into small drops, and distributing these over a large surface (beechwood shavings). The nature of the micro-organisms taking part in this manufacturing process has not yet been investigated. The use of pure-cultivated and methodically selected cultures is likely to be adopted before long.

Whilst PASTEUR, in the work mentioned above, does not explicitly maintain the theory that the oxidation of alcohol to acetic acid, brought about by acetic acid bacteria, is a purely physiological process, ADOLF MAYER expresses this opinion, the correctness of which he has confirmed. HANSEN also emphasises as a certainty the fact that the formation of acetic acid is commonly effected by the action of bacteria. PASTEUR showed that the acetic acid generated by the oxidation of ethyl alcohol is transformed, if the oxidation is continued, into carbon dioxide and water. This was recently confirmed by ADRIAN J. BROWN, to whom we owe the most thorough investigations into the chemical effects of acetic acid bacteria.

HANSEN'S researches are among the first which proved that a definite fermentation is not induced by one species of bacterium only, but by several; these researches also furnish some of the earliest experimental evidence of the fact that one and the same species can occur in very different shapes; the correctness of his results was later confirmed by ZOPF, DE BARY, and A. J. BROWN. By means of his staining experiments with *Bact.* (*Mycoderma*) *aceti* (1879), he discovered that at least two distinct species are hidden under this name, of which the one, like most other bacteria, is stained yellow by iodine, whilst the other assumes a blue coloration with the same reagent. For the former he retained the old name *Bact. aceti*, whilst the one stained blue he named after PASTEUR—*B. Pasteurianum*. The film formations on wort and beer, and likewise the growths on wort-gelatine, give a fine blue colour with tincture of iodine, or iodine dissolved in a solution of potassium iodide, whilst the growths which develop on yeast-water and meat-decoction with peptone and gelatine are coloured yellow; even very old films on beer show a yellow reaction. It is the gelatinous formation secreted from the cell-wall that is coloured blue. HANSEN discovered a third species at a later period.

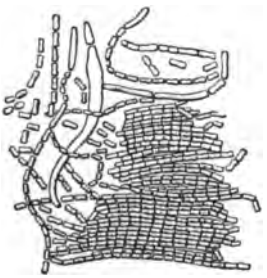

FIG. 14.—Bacterium aceti. (After HANSEN.)

These three species are characterised as follows: *Bacterium aceti* (HANSEN) (Fig. 14) forms a *slimy smooth film* on "double beer" (top-fermentation beer, rich in extract, containing 1 per cent. of alcohol) at a temperature of 34° and in the course of 24 hours. The slime is not coloured by any iodine solution. The cells of this film are hour-glass shaped rod-bacteria, arranged in chains; occasionally longer rods and threads occur, with or without swellings. At 40°-40½° C. long thin threads develop. In plate-cultures with wort-gelatine, this bacterium at 25° C. forms colonies with sharply defined edges, or, more rarely, stellate colonies, which appear grey by reflected light, bluish by transmitted light; they mainly consist of single rod-bacteria. In meat-water peptone-gelatine the colonies are surrounded by milky zones, separated from the colony by bright zones; they may later become iridescent. On sowing drops on wort-gelatine, flat, rosette-shaped colonies are formed at 25° C. in the course of 18 days. In "double beer" the temperature maximum for growth is 42° C., the minimum 4-5° C.

This species is of common occurrence both in high- and low-fermentation beers.

Bacterium Pasteurianum (HANSEN) (Fig 15) forms a *dry film* on "double beer" at 34° C., which *soon becomes wrinkled and pleated.* In young, vigorous films on beer or wort, at favourable temperatures, the slime surrounding the cells is coloured blue by any iodine solution. The cells of the film form long chains and are usually larger, especially thicker than in the previous species. The thread-form at 40-40½° C. is also a little thicker than that of B. aceti. In plate-cultures, with wort-gelatine at 25° C., the colonies resemble those of the previous species, but are a little smaller, and consist chiefly of chains. In meat-water peptone-gelatine the colonies are similar to the previous species. On sowing drops on

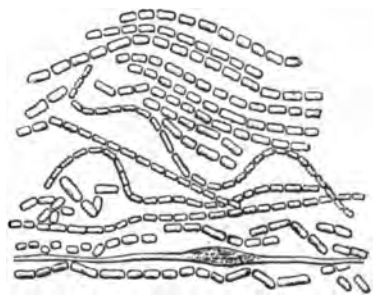

FIG. 15.—Bacterium Pasteurianum. (After HANSEN.)

wort-gelatine wrinkled colonies develop at 25° C. in the course of 18 days, which are slightly raised and present a sharp outline or one slightly jagged. In "double beer" the temperature maximum of the growth is 42° C., minimum 5-6° C.

This species is more frequently met with in high- than in low-fermentation breweries.

Bacterium Kuetzingianum (HANSEN) (Fig. 16) forms a *dry film*, on "double beer" at 34° C. which *raises itself high above the liquid, up the side of the flask.* Under the same circumstances as in the case of B. Pasteurianum the slime is coloured blue. The film consists of small rod-bacteria, which are most frequently single or connected in pairs, but seldom form chains. The thread-form at 40-40½° C. presents almost the same appearance as that of B. Pasteurianum. In plate-cultures with wort-gelatine at 25° C. the colonies are analogous to those of the previous species. They consist almost exclusively of small, single rod-bacteria. In meat-water peptone-gelatine the colonies likewise resemble those of the two previous species. On sowing drops on wort-gelatine at 25° C. colonies develop in the course of 18 days resembling those of B. Pasteurianum, but with a smooth surface, without wrinkles. On "double beer" gelatine these colonies are slimy, whilst in the two previous species they have a dry surface.

FIG. 16.—Bacterium Kuetzingianum. (After HANSEN.)

In "double beer" the temperature maximum of the growth is 42° C., minimum 6-7° C.

This species was found in "double beer."

Bacterium xylinium is essentially different from these three species. It was described by ADRIAN J. BROWN in 1888, who examined it especially from a chemical point of view. This species is used in England for making vinegar. It forms a film, the slime of which becomes cartilaginous and tough like leather, the growth gradually filling up the whole liquid. This species is essentially different from the three first described in another respect, viz.: the slimy envelope in this species

shows the *cellulose reaction*, which is not the case with the slime of HANSEN'S three species.

Acetic acid bacteria have also been described by PETERS, VERMISCHEFF, DUCLAUX, LINDNER, ZEIDLER, and HENNEBERG. ZEIDLER found a motile form in lager beers, which he named *Thermobacterium aceti*.

A species with motile cells, *Bacterium oxydans*, was described by HENNEBERG, who, according to ZOPF, discovered it on low-fermentation beer which had been standing in vessels at a temperature of 25-27° C. This species forms roundish colonies in gelatine, which later assume irregular shapes with curious ramifications. On sterilised beer it forms a delicate film consisting of separate prominences rising to a considerable height round the sides of the vessel. The film consists, when young, of cells arranged in pairs, but later, of long chains. Beer is rendered turbid by this species. In the earlier stage, when the cells are mostly in pairs, *motile cells* have been observed. At a temperature of 36° C. the growth on beer consists almost exclusively of long, uniform threads. This species also shows the irregular, swollen forms, for instance on beer at 26° C. The cells are not coloured blue by iodine. The optimum temperature for the growth appears to lie between 20° and 25° C. The higher temperature limit for the formation of motile cells was found to be 44° C. in the case of a culture 25 hours old. The temperature at which the vegetation is destroyed lies between 55° and 60° C. when moist, 97° and 100° C. when dry. The oxidation of alcohol into acetic acid has its optimum between 23° and 27° C.

HANSEN'S thorough investigation of acetic acid bacteria has assumed great importance in the general biology and morphology of bacteria, owing to the light thrown on one of the factors which cause multiplicity of bacterial forms.

Each single species of the acetic acid bacteria examined by HANSEN occur in three essentially different forms dependent on temperature, namely, *chains*, consisting of short rods, *long threads*, and *swollen forms*. If sown on "double beer," which is very favourable to their growth, the various species give at all temperatures *between about 5° and 34° C.* a growth consisting of the *chain form*, which develops well, notably at 34° C. If a

bit of this young film is transferred to fresh nutritive liquid at *40°-40½° C.*, the cells grow into *long threads* in a few hours (Fig. 17). In some species these threads can attain a length of 500 μ[1] and more, whilst the links of the chains measure only

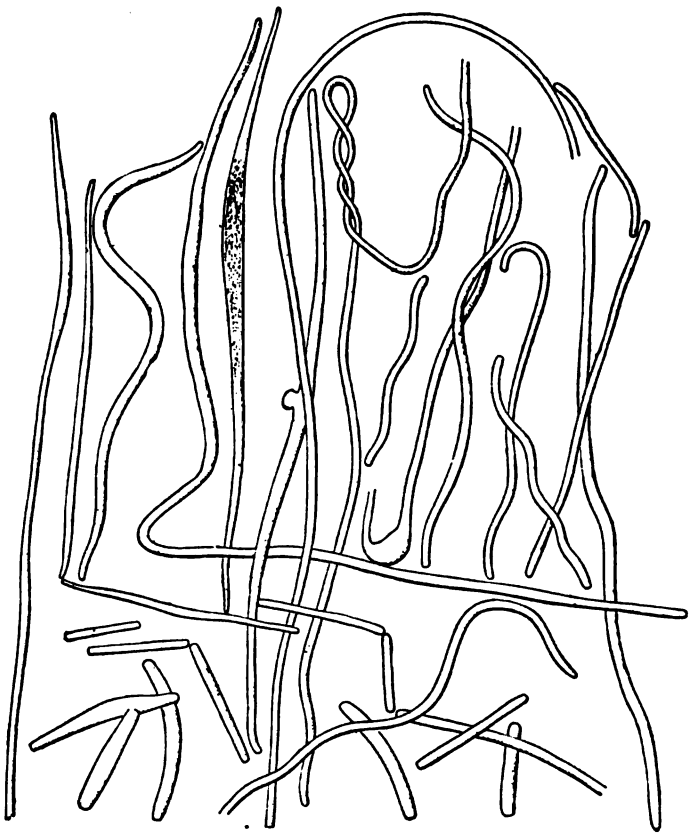

FIG. 17.—Bacterium Pasteurianum. The thread form after cultivation for 24 hours on "double beer" at 40°-40½° C. (After HANSEN.)

2-3 μ. If then this growth of long threads is placed at a temperature of *34° C.* a transformation into the *chain form* again takes place; while developing at this temperature, the long threads not only increase in length, but also in *thickness*, and that often very considerably. Thus an endless variety of *poly-*

[1] 1 μ=0·001 millimetre.

morphous swollen forms (Fig. 18) are produced. It is not till then that the threads are divided into small links, thus giving rise to the typical chains. Only the thickest parts of the swollen threads remain undivided and are at last dissolved. Thus the

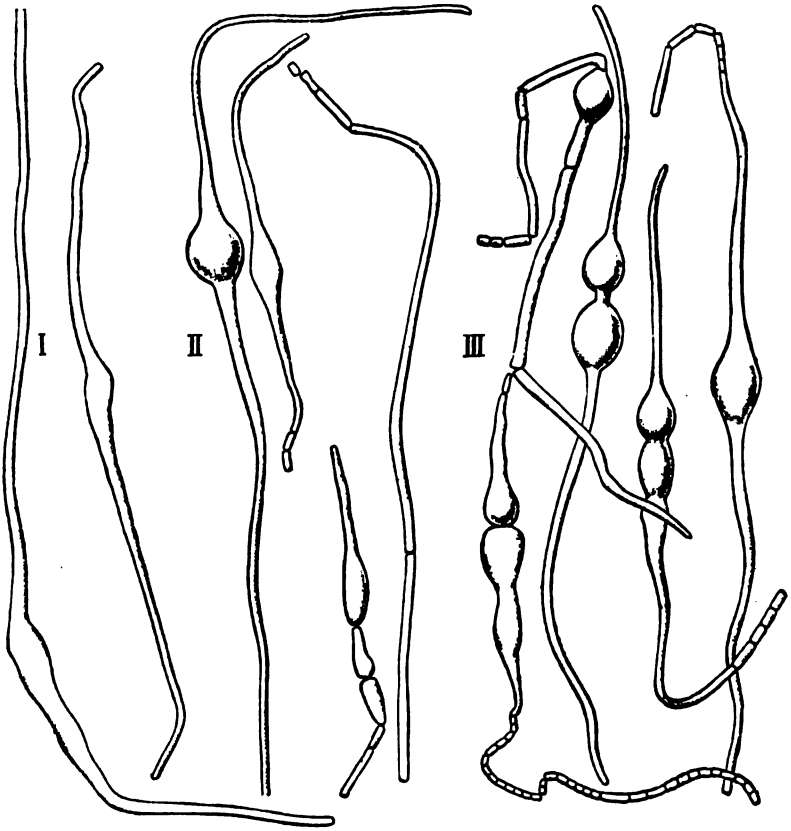

FIG. 18.—Bacterium Pasteurianum. Transformation of the thread-forms and chains after cultivation in "double beer" at about 34° C. (After HANSEN.)

swollen forms play a regular part in the cycle of changes. This cycle furnishes a striking example of the effect of *temperature* in determining the form assumed by bacteria.

The species *Bacterium aceti* and *Bacterium Pasteurianum* differ, according to LAFAR, both chemically and chemico-physiologically. In sterilised beer they give different fermen-

tation reactions. At higher temperatures *Bact. Pasteurianum* acquires a higher acidifying power than *Bact. aceti*; on the other hand, *Bact. aceti* was able to carry on a vigorous fermentation at 4° to 4½° C., whilst at this temperature *Bact. Pasteurianum* formed no appreciable amount of acetic acid. At 33°-34° C. *Bact. Pasteurianum* reached the maximum of acetic acid formation in seven days, namely, 3·3 per cent. by weight. After the maximum of acid formation had been reached, irregularly swollen cells made their appearance in the growth, which under the existing nutritive conditions may probably be considered as diseased or degenerate forms (involution forms). More thorough investigation into this question is to be desired. The forms noted by HANSEN in the cycle described above have quite a different physiological signification, in that they are then developing freely, and thus preparing the growth for the formation of new cells.

Acetic acid bacteria play an important part in the fermentation of beer, spirits, and wine. Especially in the last-named liquor they cause much harm, and if they once attain a strong development, the wine is irretrievably spoilt.

In low-fermentation breweries they usually do less mischief, as their growth requires a high temperature and an abundant supply of air. Thus, they are readily suppressed in a well-arranged lager beer cellar. HANSEN'S experiments have shown that *Bact. aceti* and *Bact. Pasteurianum* are able to exist during the whole time of storage, whether infected at the beginning or end of the principal fermentation. The contamination, however, did not manifest itself during the whole course of the fermentation either by the taste or by the smell of the beer. When the beer was drawn off into bottles and exposed to a higher temperature, the bacteria developed further; yet, when the bottles were *well corked*, the beer did not turn sour. Just the same result was arrived at when the finished beer was infected. If, on the contrary, the bottles are badly corked, the growth turns the beer sour.

In high-fermentation breweries, on the other hand, where fermentation is carried on at higher temperatures, these bacteria are capable of doing much mischief, even before the beer leaves the brewery.

To those in practice, it is interesting to note that the best known of these aërobic bacteria exert *no influence on the colour or brightness of the beer*, whilst most other bacteria cause turbidity.

Likewise in distilleries, and more especially in air-yeast factories, acetic bacteria may occur in large quantities, as shown by numerous experiments made by the author; they are most frequently accompanied by mycoderma species. A careful control of the manufacturing process from this point of view should never be omitted.

While investigating the influence of acids, especially *acetic acid on vine yeasts*, LAFAR found that each of the different acids (malic, tartaric, lactic, acetic, etc.) exerts a peculiar influence on the yeast, and not only on the proportionate amounts of alcohol and carbon dioxide produced, but also on that of glycerine; the acetic acid samples contained the smallest amount of glycerine and showed the weakest propagation of yeast. Contrary to the previously accepted view that even small amounts of acetic acid prevent alcoholic fermentation, LAFAR found that the presence of 0·27 per cent. of this acid had practically no influence on the rate of fermentation, the multiplying of the cells, or the yield of alcohol and glycerine. In must, before neutralisation, the yeast-cells were not impaired by an addition of 0·74 per cent.; but in neutralised must, after adding as much as 1 per cent. of acetic acid, 4·77 per cent. by volume of alcohol was formed, *i.e.* 60 per cent. of the maximum yield. Yeasts differ, however, considerably in their sensitiveness to the action of acetic acid. Thus, a comparison of fifteen different wine yeasts showed that all were able to carry on fermentation in the presence of 0·8 per cent. of acetic acid in a must that had previously been neutralised, whereas with 1 per cent. of acid only three were active. With regard to the propagation of cells, yeasts behave very differently with the same amount of acetic acid. LAFAR also examined the influence of this acid on the chemical activity of wine yeasts, *i.e.*, on the proportion between the amount of alcohol produced and the number of yeast-cells formed. He found that in presence of 0·88 per cent. of acid the amount of work done by one cell was *greater* in the case of ten varieties, but smaller in two

varieties, than in the presence of 0·78 per cent. Those yeasts, which are active in presence of 1 per cent. of acid, showed, under these circumstances, a decrease in work done as compared with the results in presence of 0·88 per cent.

2. *LACTIC ACID BACTERIA.*

When milk is exposed at a temperature of 35° to 42° C. it soon turns sour, a considerable portion of the acid produced being lactic acid, which is formed by the agency of various species of bacteria. When a certain quantity of lactic acid has been formed, the fermentation ceases. It will recommence, however, if the liquid is neutralised with carbonate of lime, or if a small quantity of pepsine or pancreatine is added, causing the caseïne of the milk to be dissolved.

A method often employed for inducing a spontaneous lactic acid fermentation is the following:—To a litre of water are added 100 grams of sugar, 10 grams of caseïne or old cheese, and an abundant quantity of powdered carbonate of lime. This mixture is exposed in an open vessel to a temperature of 35°-40° C. The liquid is occasionally agitated, or a current of air is passed through it. After completion of the fermentation the liquid is evaporated, when calcium lactate crystallises out, and from this the lactic acid is liberated by addition of the calculated quantity of sulphuric acid.

In addition to milk-sugar, lactic acid bacteria are also capable of fermenting cane-sugar, glucose, maltose, and various other substances. According to BOURQUELOT'S investigations, a species of lactic acid bacterium, which makes its appearance in the spontaneous souring of milk, is capable of fermenting cane-sugar without previously inverting it.

In nature, these bacteria seem to occur comparatively rarely. On the other hand, they are of frequent occurrence in the various fermentation rooms.

In breweries, lactic acid fermentation takes place in malt, especially during mashing; lactic acid is also produced during fermentation. In the Belgian beers, obtained by spontaneous fermentation, lactic acid is formed in large quantity, imparting a sharp taste to the beer; the so-called "white beers" owe

their refreshing taste to a vigorous lactic acid fermentation. In modern low-fermentation breweries every endeavour is made to exclude the lactic acid ferment, and bacteria in general, from the fermentations. In distilleries, on the other hand, lactic acid bacteria are cultivated methodically, with the view of either directly suppressing other bacteria, especially butyric acid bacteria, or, at least, bringing about the most unfavourable conditions for their growth. (See below.)

We are indebted to PASTEUR for the first important work on the subject of lactic acid bacteria. In 1858 he described the species which appears when milk spontaneously ferments. In his "Etudes sur la bière" he depicts certain bacteria growing in wort or beer in which lactic fermentation has begun (Fig. 19); he describes them as short rods slightly narrowed in the middle, and commonly occurring singly, rarely united in chains. In 1877 LISTER prepared from sour milk a pure culture of a lactic acid bacterium, which he called *bacterium lactis*.

FIG. 19.—Lactic acid bacteria. (After PASTEUR.) In order to give an idea of the size of the bacteria, some yeast-cells are figured among them.

More recently, HUEPPE found a bacterium in a spontaneous lactic acid fermentation which converts milk-sugar and other sugars into lactic acid with the simultaneous formation of carbon dioxide (*bacillus acidi lactici*). It consists of short, plump, motionless cells, the length of which exceeds their breadth by at least one half; they are united in pairs or in groups of four. In sugar solutions and less distinctly in milk they form spores, which appear as lustrous spheres attached to the end of the rods. In gelatine-plates they form whitish colonies which, as long as they are submerged, are circular, with sharp contours and uniformly dark appearance; those on the surface have lighter borders. Atmospheric oxygen is necessary for fermentation with this species. It coagulates the caseïne of milk.

In recent publications descriptions are found of a large number of lactic acid bacteria; thus, two species of micrococci have been found in saliva and the mucus of teeth; species

are also found amongst the pigment-forming bacteria, which, in addition to the production of pigments, can convert so much sugar of milk into lactic acid that the caseïne of milk coagulates; to these belong, according to HUEPPE, the famous blood-portent (*Micrococcus prodigiosus*) and, according to KRAUSE, a pathogenic form, the *micrococcus* of *osteo-myelitis*.

DELBRUECK states that ZOPF has obtained a lactic acid bacterium by preparing a mash from 200 grams of dry malt and 1000 grams of water, and keeping it for some time at 50° C. The material was then sown in a solution of milk-sugar, on the surface of which the organism formed a film. The filaments consist at first of small rods, later of both rods and cocci.

PETERS found a bacterium in leaven, which produces a typical lactic acid fermentation. In plate-cultures it forms circular colonies with concentric stratification. The rods have a rapid sinuous motion. In a neutral solution of sugar in yeast-water at 30° C., this species forms a slimy film after some time, the rods having developed into long filaments. Spore-formation has not been observed.

The so-called *Pediococcus acidi lactici*, examined by LINDNER, gives a strong acid reaction when cultivated in a neutral malt-extract solution at 41° C. Both in a solution of this kind and in a hay-decoction, which have not been sterilised, this bacterium develops so vigorously that, according to LINDNER, all other organisms are suppressed. It has been proved chemically that the acid, which is abundantly produced, consists for the most part of lactic acid. When a malt mash or malt-rye mash is maintained at 41° C., the *Pediococcus* develops vigorously, and the rod-shaped lactic acid bacteria are suppressed. In a neutral malt-extract solution, the *Pediococcus* is killed after five minutes' exposure to 62° C. In gelatine it does not thrive well; it is only in puncture-cultivations in neutral malt-extract gelatine, that very vigorous white colonies are formed below the surface. This species appears, on the whole, to thrive better when the air is excluded.

The *Saccharobacillus Pastorianus* described by VAN LAER, which occurs in the form of rods of different lengths, produces a characteristic disease ("*tourne*") in beer, which manifests

itself as follows: the liquid gradually loses its brightness, and when it is agitated filaments of a silky lustre rise from the bottom, and the beer assumes a disagreeable odour and taste. In cultures, the bacillus develops both in the presence and absence of free oxygen. In nutrient liquids it ferments the carbohydrates, and amongst them the saccharoses, without previously inverting them. Amongst the fermentation products, lactic acid, acetic acid, and alcohol are especially mentioned. The acids produced cause the precipitation of nitrogenous compounds in the liquid, and these, mixed with the bacilli, produce a cloudiness, consisting of lustrous filaments. The nutritive mixture best suited to this bacterium is an extract of malt mixed with agar and a small quantity of alcohol.

GROTENFELT has recently described some species which must be regarded as new ones; at any rate he could not identify them with those described by HUEPPE and MARPMANN. Some species were observed to yield alcohol in addition to lactic acid by the decomposition of sugar; he suggests that these species may possibly play a part in the production of the aroma of butter.

E. KAYSER carried out an extensive chemical enquiry into lactic acid fermentations, for which purpose he made use of pure cultures of growths in milk, cream, beer, must, etc. It had been previously known that the quantity of acid produced varies, and also that the proportions between volatile and non-volatile acids vary according to the species. This was confirmed by KAYSER. He also found that growths of the same species, when cultivated on the surface of the liquid, yielded larger amounts of volatile acids than cultures developed at the bottom of the liquid.

The success attained by HANSEN'S pure yeast system in the fermentation industry has, of late years, led to the adoption of the same principle in some dairies, and also in the acidification process in distilleries. A brief survey of the progress made in this direction may be attempted.

As the mash in **distilleries** does not exceed a temperature of about 70° C., so that the diastase may be preserved, many of the germs adhering to the raw materials are not killed, but are

capable of developing during fermentation, and thus not only may they utilise the nutritive substances, but also disturb the desired alcoholic fermentation; in the latter respect, the butyric acid bacteria are specially dreaded. With the view of preventing a strong development of germs injurious to the yeast, various acids have been added direct to the mash, or else **a lactic acid fermentation** has been previously carried out in a fraction of the mash. Thus a tenth part may be kept at a temperature of 50° C. till it shows about 2½ degrees of acidity,[1] corresponding to about 1 per cent. of lactic acid. This temperature is the most favourable for the desired species of lactic acid bacteria. The mash is then heated up to 70° C. whereby part of these bacteria are killed. After subsequently cooling down to about 20° C. the yeast is added. The latter is not affected by this quantity of lactic acid. After the yeast has developed sufficiently, the mixture is employed for pitching the principal mash, putting aside one-tenth of the latter for the next operation.

The acid thus introduced into the principal mash together with the surviving lactic acid bacteria act as disinfectants, besides exerting an influence on the yeast-cells, both direct and by reacting on the nutritive substances.

If the whole process is not carried out with due care and regularity, the yield of alcohol will suffer, and infection with foreign ferments may take place. This happens frequently at the beginning of a distilling season, after most of the lactic acid bacteria existing in the fermentation room have died. The attempt has been made to remedy this evil by isolating the bacterium present in an acid mash which had undergone a good normal acidification; this bacterium was developed into a mass-culture and introduced into the new mash, which was then left for acidification. In this manner LAFAR isolated from the acid mash of a distillery a bacterium which differs from the milk-souring species, and named it *Bacillus acidificans longissimus*, on account of its frequent occurrence as long filaments. The experiments hitherto made with this species on the large scale by BEHRENS and LAFAR have yielded good results. LEICHMANN and the author have also prepared pure cultures of the

[1] *i.e.*, to neutralise 20 c.c. mash 2½ c.c. normal caustic soda solution are required.

lactic acid bacteria occurring in mash. It is not yet known whether one single species always predominates or whether several species are active in spontaneous acidification.

Since the year 1890 methodically selected species of bacteria have been made use of in a similar way in **dairies** for the purpose of bringing about a regular and certain acidification of the **cream** used in the manufacture of butter. The progress made in this field is associated with the researches of STORCH, WEIGMANN, QUIST, and others. The pure culture selected is added to skim-milk previously heated to 60°-70° C., and the culture is allowed to develop at about 15° C. After standing 24 hours, this mass-culture is fit for use. In order to render the cream which is to receive the culture as free from germs as circumstances permit, it is first cooled down to a low temperature, and then quickly warmed up to about 20° C., or else it is pasteurised. For a period of 24 hours the mass-culture is allowed to develop in cream before churning is commenced.

Among the forms isolated by STORCH (Copenhagen, 1890) from butter, acidulated cream, and butter-milk, the *Coccus*

FIG. 20 a.—Lactic acid bacteria. (After STORCH.)

form seems, according to WEIGMANN, to be the most frequent species and the one best suited to the acidification of the cream. It occurs in a large number of varieties, which, according to their main characteristics, may be distributed into two groups:

one including those which give more especially a pure and mild taste, and another embracing those which yield more especially a product possessing great keeping powers. Morphologically the growths are distinguished from each other by the fact that some are connected in chains, others not (Fig. 20 *b*, *a*); the latter are of the most frequent occurrence and widest range in nature. These forms bear a certain resemblance to PASTEUR'S "ferment lactique." The species represented in Fig. 20 *a* was isolated by STORCH from a sample of butter having a pure and full aroma. It forms in gelatine small globular colonies of a pure white colour and with a smooth surface. In milk and whey it occurs in oval or globular forms. These lactic acid

FIG. 20 *b*.—Lactic acid bacteria. (After STORCH.)

bacteria display a vigorous fermentative activity, even at as low a temperature as 20° C.

QUIST has cultivated another species, which has been employed with success in practice. It occurs both as micrococcus and in other forms, according to the different nutrient media in which it is cultivated. In gelatine it forms small, circular, slowly-growing colonies of a whitish-yellow colour. In puncture-cultivations spherical colonies arise throughout the puncture-channel, and in streak-cultures this organism forms a continuous streak with wavy borders. It was prepared from a sample of butter of remarkable aroma and durability.

On the other hand, several species of bacteria have been discovered in recent years, which cause diseases in milk and butter. Thus SCHMIDT-MUELHEIM found a micrococcus which occurs in the form of intertwined chains, and causes the milk to turn viscous; another species, discovered by RATZ, possesses the same property and also produces a vigorous lactic acid fermentation; other slime-forming species have been described by ADAMETZ, DUCLAUX (*Actinobacter*), LOEFFLER, GUILLEBEAU, STORCH, and LEICHMANN. WEIGMANN isolated a species which imparts a bitter taste to milk and secretes a ferment which dissolves caseïne. JENSEN likewise found several species which cause marked abnormalities in the taste of milk and butter, among them especially *Bacillus fœtidus lactis*, having the form of thick, moving rods, of varying length, some almost like micrococci. STORCH proved that the disagreeable taste of tallow sometimes noticeable in butter is caused by a certain species of bacterium, which acidifies and coagulates milk.

The "breaking" of milk in the cheese factory, in which process the caseïne is precipitated, is effected in practice by means of *rennet*, an enzyme secreted by certain glands in the stomach of different animals. The same enzyme occurs in a few plants, as for example Pinguicula, Ficus carica, and has recently been discovered in several bacteria. Thus CONN (1892) isolated such bacteria in pure cultures. Especially among the so-called "potato bacilli," bacteria of this class occur. Their enzyme, however, differs in certain respects from that of rennet. Also the renowned *Micrococcus prodigiosus* and the *Bacterium coli commune* contain the same enzyme.

The bacteria and other fungi active in the *ripening of cheese* were studied by ADAMETZ, DUCLAUX, FREUDENREICH, WEIGMANN, and others. Many of these bacteria, which in part exercise a peptonising action on caseïne, can, when occurring in large numbers, impart a definite character to the ripening process, giving rise to aromatic substances which determine the sort of cheese produced.

3. *BUTYRIC ACID BACTERIA.*

When milk which has stood for some time, in which lactic acid bacteria have developed, is neutralised by the addition of calcium carbonate (chalk), so that calcium lactate is formed, it will, as a rule, undergo a butyric fermentation. PASTEUR showed in 1861 that this fermentation is brought about by particular micro-organisms which are able to live without air (*"vibrions butyriques"*). This spontaneous butyric acid fermentation takes place most vigorously at 35° to 40° C. Starch, dextrin, cane-sugar, and dextrose are likewise decomposed by the butyric acid ferments, and these fermentations are of frequent occurrence, as the bacteria belonging to this group are very widely distributed in nature. Allied species doubtless play a part in the ripening of cheese, discussed above. In order to induce a butyric acid fermentation, FITZ recommends the employment of a mixture of 2 litres of water, 100 grams of potato-starch or dextrin, 1 gram of ammonium chloride, the ordinary nutrient salts, and 50 grams of chalk; this mixture is to be maintained at 40° C. BOURQUELOT recommends exposing slices of raw potatoes standing in water for two or three days at a temperature of 25° to 30° C. At 35° C. this fermentation appears to be still more vigorous

The most important products of the butyric acid fermentation are butyric acid, carbon dioxide, and hydrogen.

In the saccharine mashes of breweries, distilleries, and pressed-yeast factories, species of butyric acid bacteria always occur, and if the mashes are maintained for a lengthened period at certain temperatures, these bacteria develop very rapidly and exercise a retarding influence on the alcoholic ferments. If butyric acid occurs to any extent in beer, it will acquire a very unpleasant taste.

According to PASTEUR'S experiments, the butyric acid ferment can perform its functions without access to the free oxygen of the air. More recent investigations have shown, however, that many butyric acid bacteria exist which not only yield different fermentation products, but also behave differently with regard to free oxygen, in that some are not capable of developing when the latter is present,—so-called

anaërobic species,—whilst others multiply and induce butyric acid fermentation when they have access to oxygen,—aërobic species.[1]

One of the first species to be minutely described is

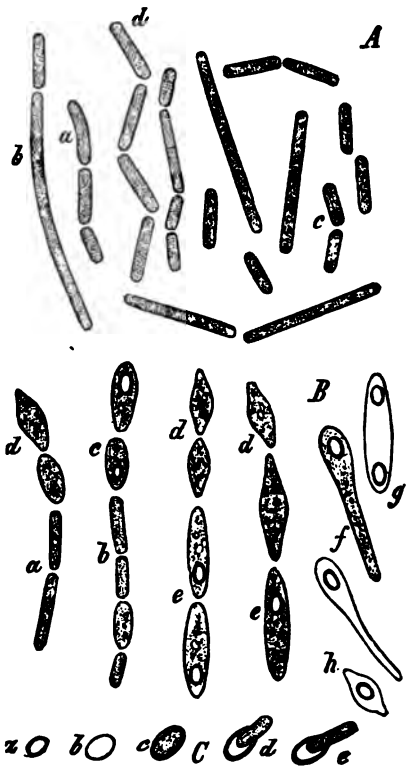

FIG. 21.—Clostridium butyricum Prazm. (after PRAZMOWSKI): *A*, vegetative state; *c*, short rods; *d*, long rods; at *a* and *b*, rods and filaments curved like vibriones; *B*, formation of "resting-spores" (Dauersporen); *b*, *d*, rods, previous to, *c*, *e*, during, *f*, *g*, *h*, after the formation of resting-spores; *c*, of an elliptical; *d* and *h*, of a lemon-like; *e*, *g*, of a spindle-like; *f*, of a tadpole-like shape; at *a*, rods still in their vegetative state; *C*, germination of resting-spores: the spore *a* expands into *b*; *c* shows the differentiation of the membrane into exo- and endosporium. The contents surrounded by the endosporium issue from the polar fissure of the spore in the form of a short rod (*d*), which appears prolonged at *e*.

PRAZMOWSKI'S *Clostridium butyricum* (*Bacillus butyricus*, Fig. 21). It occurs in the form of short and long rods, which may

[1] The pure cultivation of anaërobic bacteria may be carried out, for example, in nutritive gelatines or agar, in well-filled test-tubes, these bacteria developing in the bottom layer. A better procedure consists in removing the air

be either straight or somewhat curved. The rods are seen in brisk movement, and under a strong magnifying power they are found to be covered with a large number of cilia. Before the formation of spores in the rods, the latter swell and form peculiar spindle and lemon-shaped, elliptical, or club-like forms (see fig.); at the same time it is remarkable to note that they are coloured blue by iodine. On germination the spores burst their outer envelope, and the germ filament grows in the same direction as the longitudinal axis of the spore. *Clostridium butyricum grows most readily at a temperature of about* 40° C., and rapidly predominates in a sugar solution if the lactic acid ferment has previously converted a portion of the sugar into lactic acid. This species is decidedly anaërobic.

FITZ has described a species belonging to the aërobic organisms. It is a bacillus of a short cylindrical form, which is not coloured blue by iodine, is motile in a moderate degree, and forms no spores. It ferments all carbohydrates, with the exception of starch and cellulose.

HUEPPE has likewise described a species found in milk, and occurring in the same forms as the species discovered by PRAZMOWSKI, but it proved much less sensitive towards oxygen, and must therefore be classed with the aërobic species.

GRUBER found associated under the name of *Clostridium butyricum* three well-defined species, two of which are exclusively anaërobic. One of the latter species consists of straight or slightly-curved rods, which become spindle- or

from the test-tube by means of an air pump, the tube being kept in water at 30-35° C.; it is then closed or hermetically sealed. Another way is to pass a current of hydrogen through the nutritive liquid containing the growth in the test-tube, which is closed hermetically as soon as the atmospherical air has been expelled; then the glass is rotated on its longitudinal axis until the gelatine has coagulated and the inside of the glass is coated by it; in this gelatine coating the colonies gradually make their appearance. Substances that absorb oxygen may also be used, such as pyrogallic acid (1 gram of this acid in 10 c.c. of 10 per cent. caustic potash solution), the open test-tube or the plate-culture being placed in an air-tight tube or vessel containing the reagent; or, again, the cultures may be covered with paraffin, vaseline, oil, plates of mica, or the like. An addition of grape-sugar and slight quantities of sodium formate or the sodium salt of indigo mono-sulphonic acid to the nutritive substance renders the medium particularly favourable for these bacteria.

barrel-shaped during the formation of spores. In nutrient gelatine it forms colonies which, when seen in transmitted light, appear blackish-brown or black. The second species consists of strongly-curved vegetative rods, at the end of which the spores are formed; it forms yellowish or yellowish-brown colonies. The third species is also capable of growth and of causing fermentation in the absence of oxygen; its development is, however, decidedly assisted by the presence of oxygen, and it is only then able to produce spores. The vegetative rods are cylindrical; with the formation of spores the rods become spindle-shaped, and in the centre of the spindle the large spore is formed. The colonies in nutritive gelatine are of a yellow colour. All three species form butyric acid and butyl alcohol.

A very interesting butyric acid bacterium is the *Clostridium Pasteurianum*, closely studied by WINOGRADSKY, which possesses the power of directly absorbing and causing the combination of *the free nitrogen* of the air, whereas combined nitrogen has no nutritive value for this bacterium.

In imperfectly sterilised milk BAIER and WEIGMANN discovered two species of butyric acid bacteria, which have their optimum at 30° C., and are not strictly aërobic. They dissolve the caseïne of milk in the course of some days, and impart to the liquid an objectionable, putrid odour.

According to FITZ the spores of butyric acid bacteria can withstand the temperature of boiling water for a period of time which is naturally dependent, as in all cases, on their condition and on the nature of the substratum; FITZ gives three to twenty minutes as the limits. They can, however, be killed by a lower temperature, if continued long enough; thus they are killed by being heated for six hours at 90° C. in a solution of grape-sugar; but in glycerine, at the same temperature, only after six to eleven hours.

Thus butyric acid fermentation, like lactic acid fermentation, is not produced exclusively by one species. When butyric acid fermentation occurs in distilleries, breweries, and pressed-yeast factories, bacteria are frequently found which are entirely different from those described above.

Clostridium butyricum, and various other species, are capable of dissolving cellulose, and therefore play an important part in

cellulose fermentation, which is employed in various branches of industry.

A bacterium which, among other substances, produces butyric acid is the *Bacillus lupuliperda* examined by BEHRENS, which occurs very frequently on hops, and is the cause of the *spontaneous ignition of moist hops*. The growth consists of movable cocci and short bacilli, which render gelatine liquid. In a medium not containing sugar this fungus produces an abundance of ammonia derivatives, especially trimethylamine (odour of pickled herring); in the presence of sugar the solution soon becomes sour, and butyric acid is formed. This species seems to have its abode chiefly in the soil, and bears much resemblance to the *Bacillus fluorescens putridus* described by FLUEGGE.

4. *ALCOHOL-FORMING BACTERIA.*

Among the fermentation-products of several species of bacteria, alcohol occurs. The first known of these species

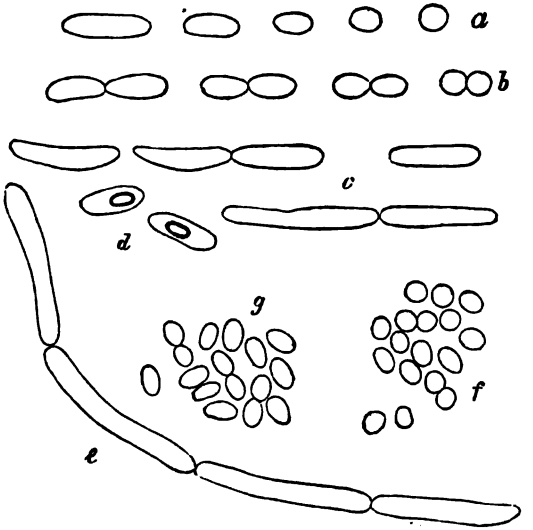

FIG. 22.—Bacillus Fitzianus (after H. BUCHNER.) *a*, *b*, *f*, *g*, cocci and short rods; *c*, *e*, bacilli; *d*, rods with spores.

is the *Bacillus Fitzianus*, discovered by FITZ in hay-infusion

(prepared cold), and later more accurately described by H. BUCHNER (Fig. 22), which occurs with coccus and bacillus forms. In a nutritive solution containing glycerine it ferments this latter substance, forming chiefly ethyl alcohol. The *Bacillus ethaceticus*, discovered by P. FRANKLAND, ferments glycerine, mannite, or arabinose, forming ethyl alcohol in conjunction with acetic acid. Finally, the *Bacillus pneumonia* of FRIEDLAENDER may be mentioned, which is not only a pathogenic organism, but also, in nutritive sugar solutions, forms ethyl alcohol, acetic acid, and other products.

As stated above, one of the fermentation products of the three species of butyric acid bacteria, described by GRUBER, is butyl alcohol. BEIJERINCK includes all bacteria giving a butyl alcohol fermentation under the designation of *Granulobacter*, because in the absence of oxygen they form granulose, and are then coloured blue by iodine. The species described by this author are probably identical with the butyric acid bacteria of PRAZMOWSKI, FITZ, and GRUBER.

5. *KEPHIR-ORGANISMS AND GINGER-BEER PLANT.*

The so-called "*Kephir*," on which the investigations of KERN have thrown some light, is an effervescent, alcoholic and sour milk, which is prepared by the inhabitants of the Caucasus from cows', goats', or sheep's milk. It is prepared by adding a peculiar ferment, "kephir-grains," to milk. These are white or yellowish, irregularly-shaped, uneven grains, about the size of a walnut and of a tough gelatinous consistency, and when dried become cartilaginous and brittle. The essential part of these grains consists of rod-like bacteria, which are connected in threads and have developed gelatinous membranes. KERN calls this bacterium *Dispora Caucasica*. According to BEIJERINCK this species produces in milk-sugar, cane-sugar, glucose, and maltose a direct lactic acid fermentation. Besides the bacteria, there occur in the kephir-grains various yeast fungi and, frequently, moulds.

In the preparation of kephir a little milk is first poured on the grains and allowed to stand for twenty-four hours at about 17° C.; the milk is then poured off, and the grains

preserved for future use. This milk is now mixed with fresh milk, and poured into bottles which are corked, or into leather sacks which are tied; the fermentation, if the liquid is frequently shaken, is completed in two to three days. It now contains about two per cent. of alcohol. This result is probably brought about by the simultaneous action of the *Dispora* and the yeast cells in combination with the lactic acid ferments which are probably always present in milk. These ferments convert a portion of the milk-sugar into lactic acid; the alcohol and a part of the carbon dioxide result from the action of the yeast cells. Then, as the fermented milk, according to some investigators, contains less coagulated caseïne than ordinary sour milk, it may be assumed that the *Dispora* is also able to partly liquefy (peptonise) the coagulated caseïne, perhaps with the help of the gelatinous mass secreted by the bacterium, which is found in the kephir-grains, but is not present in the fermenting milk. According to recent investigations of HAMMARSTEN, however, the amount of caseïne does not appear to decrease, but a part of it undergoes certain alterations, inclusive of physical change, in consequence of which it becomes more finely flocculent. The deviation in these results may possibly originate in the different biological composition of the kephir-grains selected.

FREUDENREICH constantly found *Dispora Caucasica* in a number of kephir samples, which readily developed on milk-agar plates and in milk-sugar broth at a temperature of 35° C.; the bacilli frequently have resplendent points at both ends, and FREUDENREICH presumes that this phenomenon coincides with the formation which KERN regarded as spores; unmistakable spores, however, were not found.

Further, there occur in all samples two lactic acid coccus forms and a yeast-species. One of the cocci (*Streptococcus a*) forms diplococci and chains, and produces in milk-sugar gelatine large, white colonies, which show a coarse granulation near the border; the best temperature for the growth of this species is about 22° C.; it coagulates milk most rapidly at 35° C., and contributes essentially to the production of a sourish taste and fine flocculent appearance. The other coccus (*Streptococcus b*), which likewise forms diplococci and chains,

occurs in smaller colonies than *a*, and, in contrast with the latter, grows well at higher temperatures, and forms more acid than *a*, but does not coagulate milk. If this species is transferred, together with the kephir-yeast, to milk-sugar broth, the fermentation is more vigorous than if the bacteria alone are inoculated; FREUDENREICH therefore presumes that Streptoccus *b* splits up the milk-sugar, and that the fermentation of the latter is rendered possible by this previous decomposition. The *kephir-yeast* discovered by him shows a remarkably strong growth and a weak fermentation in beer-wort, but does not appear to produce any fermentation in milk or milk-sugar broth. The growth consists of oval cells (3-5 μ long, 2-3 μ broad); it forms neither a film nor spores; the optimum temperature of this species lies at 22° C.

In the course of his experiments, FREUDENREICH succeeded in producing a liquor resembling kephir, for which purpose he inoculated a mixture of the four species, detailed above, in milk, and, after a lapse of some days, introduced a small portion of this sour, coagulated milk, which had been repeatedly shaken, into sterilised milk; he therefore concludes that these four species, through their symbiosis, are able to bring about the kephir-fermentation. He could not observe any synthesis of kephir-grains by means of the four species, and it is not yet clear what part Dispora Caucasica plays in the whole process; moreover, it appears to be highly probable that species of bacteria, other than the two coccus-forms described by FREUDENREICH, in addition to other budding fungi, are active in the process. It may be deserving of special notice that in the author's laboratory it has been proved that in Russian kephir-grains a genuine *Saccharomyces* occurs *which ferments milk-sugar independently*, whereas all previous investigators only found budding fungi incapable of spore formation.

In some parts of North America a ferment resembling kephir-grains is used in the fermentation of saccharine liquids. According to MIX' researches it contains a yeast-species which agrees with the one described by BEIJERINCK and also a Bacterium which resembles KERN'S Dispora.

If one of the kephir-grains is allowed to remain in milk, it

will grow very slowly and only attain, according to DE BARY, to double its size after the lapse of several weeks. He considers it probable that under such conditions single *Dispora* cells separate and give rise to new kephir-grains.

According to the mode of preparation published by A. LEVY, kephir can also be obtained without the addition of KERN'S ferments. When milk, which is turning sour, is repeatedly and violently shaken, an effervescent alcoholic kephir-like drink is obtained, which, as regards taste, etc., does not perceptibly differ from kephir prepared with kephir-grains.

The *Ginger-beer Plant*, which presents morphological resemblances to the *Kephir* ferment, has been examined from a botanical and biological point of view by Professor MARSHALL WARD. If this ferment is introduced into saccharine solutions to which ginger has been added, it transforms them into an acid effervescing beverage, ginger-beer. When fresh, it occurs as solid, white, translucent lumps, of irregular shape, brittle like dried jelly, their size varying from that of a pin's head to that of a large plum. It induces an alcoholic fermentation in the saccharine solution, which at the same time becomes viscous. MARSHALL WARD isolated the numerous micro-organisms existing in the masses described above, and gave accurate descriptions of a series of yeast-fungi, bacteria and moulds, among which two organisms proved to be essentially concerned in the fermentation of ginger-beer. One of these is a Saccharomyces, belonging to the ellipsoid group of this genus, and probably originating from the ginger and brown sugar commonly employed; the author has named it *Saccharomyces pyriformis*. It inverts cane-sugar, actively ferments the products, and forms a white pasty deposit at the bottom of the vessel. It yields spores on gypsum blocks at 25° C. in 40 to 50 hours; it also forms spores on gelatine. In hopped wort it induces a rather feeble fermentation, and forms a film on the surface; many of the cells in this film are pear or sausage-shaped.

The other constantly occurring and essential form is a Schizomycete, *Bacterium vermiforme*, which, according to Professor WARD, originates from the ginger, and is active in the lactic acid fermentation. It is a peculiarly vermiform organism, enclosed in clear, swollen, gelatinous sheaths, and imprisoning

the yeast cells in brain-like masses formed by its convolutions. It is the swollen sheaths of this organism which constitute the jelly-like matrix of the "plant." It also appears without the sheaths, and in all the various growth-forms which we meet with among the bacteria. It is a markedly anaërobic bacterium. The gelatinous sheaths are only developed when the saccharine liquid is acid, and free from oxygen.

Of the other organisms which occur in the ginger-beer plant, a *Mycoderma* species and *Bacterium aceti* were found in all the specimens examined, and a variety of other bacteria and fungi occurred as casual intruders.

MARSHALL WARD has proved experimentally that *Saccharomyces pyriformis* and *Bacterium vermiforme* are the only two essential species in the ginger-beer fermentation, since it was only by inducing a fermentation with these two species that he was was able to produce an effect similar to that obtained when the ordinary ginger-beer plant is employed. But it is only when both species develop together in the liquid that they bring about this result, and his experiments indicate that the relations between the yeast and the bacterium are those of true symbiosis, so that the two species form a lichen-like compound organism, which induces a "*symbiotic fermentation.*"

6. *SLIME-FORMING BACTERIA.*

Among the various species of slime-forming bacteria there are several which are of peculiar interest in the fermentation industries, as they occur in wine and fermenting wort, causing morbid changes. By analogy, this slime formation may be regarded as a phenomenon closely related to the commonly occurring zoogloea formation of certain bacteria. In the case of some species the slime is, however, regarded as a product of the decomposition of sugar, and not as a substance separated from the organism itself.

In the viscous fermentations examined by BECHAMP a kind of gum termed viscose was formed together with carbon dioxide and, frequently, mannite.

In his "Etudes sur la bière" (Plate 1, Fig. 4) PASTEUR describes bead-like chains of spherical organisms, which render

wine, beer, and wort so viscous that they can be drawn out in threads.

KRAMER has described a *Bacillus viscosus sacchari*, which in a short time converts a cane-sugar solution into a slimy tough mass. Another species, which makes beet juice slimy, at the same time producing an ethyl alcohol fermentation, was described by GLASER under the name of *Bacterium gelatinosum betæ.*

CRAMER isolated a *Bacterium viscosus vini*, which was cultivated in sterile wine, air being excluded. Wines into which this growth had been inoculated grew thick in the course of six to eight weeks. This species grows best at 15°-18° C., and seems to die at as low a temperature as 30° C.

In Berlin "Weissbier" (white beer), which had turned ropy, LINDNER found a strong development of a certain *Pediococcus.* The disease could be produced by adding pure cultures to sterilised white-beer wort. On the other hand, this organism had no action on hopped beer-wort or low-fermentation beers.

In *ropy* Belgian beer VAN LAER found the cause of this disease to be small, very thin rods (1·6 to 2·4 micro-millimeters long), which were partly isolated and partly united in pairs by means of a zooglœa-like substance. When added to beer-wort, this first became turbid, and afterwards ropy. On meat decoction with gelatine these rods gave colonies with concentric rings of different colours and with a hollow in the middle; streak cultures give broad, white bands, with a sinuous border; puncture-cultivations give a white stripe, soon extending to the bottom of the glass; the gelatine forms fissures which become filled with the growth, while at the same time a speck is formed on the surface. Experiments carried out with pure cultures of this bacterium in beer-wort have shown that one and the same form includes several species, which have a somewhat different action on wort. They are all included under the name *Bacillus viscosus.* If sterilised wort is infected with this bacterium and alcoholic yeast added after the lapse of some hours, the liquid becomes viscous. If the wort is infected with a mixture of absolutely pure yeast

and bacteria, the disease will develop in a varying degree, according to the proportion of bacteria. If, however, these are only added after the completion of the primary fermentation, the disease will not appear at all. The greater the proportion of *nitrogenous* matter in the liquid, the sooner it will become viscous; even liquids which do not contain sugar can be made ropy by these species; when the nutritive liquid contains much sugar, the fungus develops very feebly, and in pure sugar solutions the phenomenon does not make its appearance at all.

VANDAM found in English beers an aërobian *Bacillus viscosus*, which occurs as small rods, single or united in chains consisting of two, three, or more links; in the centre of these rods spores are formed. This bacillus develops best at about 30° C., and produces a slimy mass in brewers' wort, which under the microscope proves to consist of zoogloea formation. After the lapse of some time the liquid has the consistency of albumen. No gas is evolved, but the liquid acquires a peculiar odour. On meat-juice gelatine mixed with sugar and on wort gelatine the growth develops freely. The viscosity of the liquid does not seem to depend on the quantity of nitrogenous matter present. This species is incapable of producing disease in beer unless it is thriving well and is introduced in large quantities into the wort before or during pitching. Like the forms discovered by VAN LAER, this species ferments milk-sugar; and, according to VANDAM, it is easy to detect it in yeast, even in traces, simply by introducing a sample of the latter into nutritive liquid containing milk-sugar, a growth of this species soon making its appearance in the upper part of the liquid.

BROWN and MORRIS mention a *Coccus* form which also seems to produce ropiness in English beers. This species occurs as diplococcus and in tetrads, and gives yellow wax-like colonies on meat-broth gelatine. The disease made its appearance in the beer after a lapse of six to eight weeks; but it was not usually possible to produce it by inoculation with pure cultures of the species in sterile beer. Close to the fermentation room there was a pork-butcher's premises, in which putrefying matter had accumulated; after this had

been removed and the soil dug and cleaned, the disease disappeared.

FELLOWES also analysed several English beers affected by this disease, and isolated the bacteria detected; but by inoculation of the pure cultures in beer he did not succeed in preparing a beer containing these organisms and showing

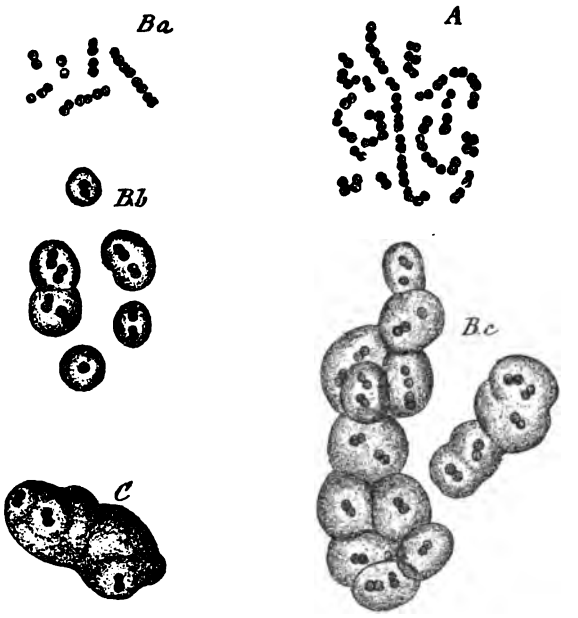

FIG. 23.—Leuconostoc mesenterioides Cienkowski (after ZOPF): *A*, cell cluster of the variety with no envelope, taken from a potato cultivation; *B*, series showing the development of a cultivation grown in gelatine, free from sugar; *B a*, has no envelopes; *B b*, the same after 24 hours' growth in a solution of molasses, the envelopes are already seen but are not strongly developed; *B c*, after 48 hours' growth in molasses, the envelopes are more strongly developed and partly encased in each other; *C*, a small gelatinous mass from which the cells have been expelled.

a viscosity corresponding to that of the sample from which they came.

The so-called *frog-spawn fungus* (*Leuconostoc mesenterioides*) was investigated by CIENKOWSKI and VAN TIEGHEM, and more recently by ZOPF and LIESENBERG (Fig. 23). Both the European form and the variety found by WINTER in Java occur spontaneously in beet-root sap, and in the molasses from the manufacture

of sugar, in which they form large slimy masses ("frog-spawn") and multiply vigorously. The fungus forms chains of cocci, two of which are always more closely united; unlike earlier observers, ZOPF found that these cocci present no differences with reference either to their morphology or physiology; spore formation could in no case be proved. Consequently, the analogy which was formerly assumed to exist between this fungus and the algal genus *Nostoc* (implied in the name *Leuconostoc*) falls through.

Under certain conditions the cells are surrounded by a strong gelatinous envelope, which consists of a mucilaginous carbohydrate, the so-called *dextran*. This formation—a product of assimilation—only occurs in the presence of cane and grape-sugar, and not in solutions of milk-sugar, maltose and dextrin, because these carbohydrates cannot be assimilated: the same applies to glycerine. In presence of the last-named substances, or in potato-cultures, the species develops quite a different form, in which the gelatinous envelope is completely absent.

The *Leuconostoc* ferments grape-sugar, cane-sugar (after previous inversion), milk-sugar, maltose, and dextrin, with production of gas and acid. The acid was shown to be lactic acid. The fungus secretes an enzyme which inverts cane-sugar; but no other enzyme could be detected.

Especially characteristic of this fungus is its power of resisting high temperatures, the *younger* growths possessing this power in a higher degree than older cultures. Thus a growth will stand gradual heating to 86°-87° C. effected in the course of some minutes. The most favourable temperature for development lies between 30° and 37° C.

It is also remarkable that the growth and fermentative action of the fungus are very favourably affected by the presence of considerable quantities of calcium chloride.

Finally, a *Streptococcus* may be mentioned, which in the manufacture of *Edam cheese* is deliberately added to the milk, to make the latter ropy. This cheese gets its peculiar character from this treatment.

7. *BACTERIA EXERCISING AN INVERTING, DIASTATIC, OR PEPTONISING ACTION.*

Bacteria play a great part in the formation of soluble chemical ferments. This constitutes one of the chief means by which these organisms produce such marked effects in the economy of nature.

According to HANSEN, many species of bacteria which generally occur in *beer* secrete *invertive ferments.* Amongst these species are a number of bacteria which exhibit an invertive action on a pure cane-sugar solution, but lose this property when yeast-water is added.

FERMI and MONTESANO found that, *e.g.*, *Bac. megaterium*, *Proteus vulgaris*, and *Bac. fluorescens liquefaciens* invert this sugar in neutral broth containing 4 per cent. of cane-sugar. Several of these bacteria, however, lose their power of inversion if the broth is rendered alkaline, whilst most of them are uninjured in slightly acid broth. In broth without sugar and in media containing no albumen such bacteria produce invertine; thus almost all the species that were examined formed invertine in a nutritive salt solution containing glycerine. The invertine, produced by these bacteria proves to be a soluble enzyme, which is destroyed at temperatures differing according to the species, but is always more resistant during its action on cane-sugar than in a dissolved state; it is very sensitive to acids and alkalies, and especially to mineral acids and potash.[1]

Similar properties to those discovered by HANSEN were observed by WORTMANN in the case of bacteria which develop *diastatic ferments.* He found these on putrefying beans and potatoes, and grew the cultures in mixtures of nutritive salts and wheat-starch. MARCANO also found a species which exer-

[1] According to ADR. J. BROWN, the *Bacillus subtilis* (Hay bacillus), which occurs everywhere, also belongs to this class of bacteria. He states that this bacterium does not develop in beer or wort of normal acidity, even if the latter is very low. In a neutral hay-infusion containing 5 per cent. of dextrose it oxidises the sugar; it also inverts cane-sugar and completely oxidises it.

cises diastatic action and frequently occurs in the outer husk of maize. PETERS found a bacillus in leaven which brought about the solution of starch. In ordinary gelatine-plate cultures this fungus forms peculiar curved colonies, consisting of long filaments about 0·5 μ thick. In beer-wort the bacillus forms rods which exhibit very active movement, and gradually produce a film on the surface. The spores are rod-like.

Bacteria have also been found which cause the solution of albuminoid substances, for example, *Micrococcus prodigiosus*. The peptonising species which are active during the ripening of cheese should be included in this class.

8. *SARCINA FORMS.*

In addition to *Pediococcus acidi lactici*, described above, there occurs in fermenting liquids a number of spherical bacteria, the life-histories of which are but imperfectly known. Both in bottom- and top-fermentation (especially in distilleries and pressed-yeast factories) different varieties of *Micrococci* occur, the injurious action of which is strongly emphasised in the technical journals. This has, however, only been satisfactorily demonstrated by direct experiment in a single case (see section on "Slime-forming bacteria"). In bottom-fermentation lager-beer these forms appear as small, more or less spherical, clear grey bodies, sometimes isolated, sometimes arranged in groups, generally in groups of four. They were described by HANSEN under the name of *Sarcina* (Fig. 24).

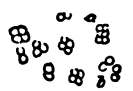

FIG. 24.—Sarcina.

Organisms belonging to this group are found in very different localities. The sources from which the individual species are derived are, however, not yet known.

REINCKE found that lager-beer infected with such bacteria soon yields a considerable sediment, and develops a bad odour and taste. Berlin white beer often assumes a red colour; it is then found to be infected with many *Sarcina* forms, the growth of which increases considerably after a few days at a somewhat higher temperature; temperatures between 10° and 14° C. are stated by REINCKE to be particularly favourable. However, he rightly emphasises the fact that it is not certain

whether *Sarcina* or the rod bacteria, which are also present, are the actual cause of the disease; it is only known that in red beer the presence of *Sarcina* is a symptom of abnormal conditions; whether it is the cause or the result can only be decided by direct experiment.

In the fresh residues from the distillation of spirit, which are employed as fodder, BRÄUTIGAM found a sarcina-like micrococcus, which possesses pathogenic properties. It has not yet been determined by direct experiments whether the so-called "malanders" or "greasy heels" of domestic animals is caused by this organism.

LINDNER examined a series of sarcina-like organisms, and contributed largely to our knowledge of their life-histories. The so-called *Pediococcus cerevisiæ* appears in cultures in the form of *cocci*, *diplococci*, or *tetrads*. Cultures made on meat broth with peptone gelatine, partially covered with thin plates of gypsum, showed that free access of air is favourable to the growth of colonies of this bacterium; during the first few days all the colonies were found to be colourless; subsequently a yellowish, or yellowish-brown tinge appeared. The gelatine was not liquefied. Streak cultures of this organism on meat-broth gelatine gave a greyish-white, moist streak, with almost smooth edges, strongly iridescent in thin layers. In puncture-cultivations it developed throughout the length of the puncture, forming a white tuft on the surface of the gelatine, which spread out like a leaf. On boiled slices of potato this species thrives but poorly; in older cultures of this kind peculiar involution forms appear. In meat-broth gelatine the organism was killed by eight minutes' heating at 60° C., but not at 50° to 55° C. after the lapse of 12 minutes. In hopped beer-wort it yields a sediment, and subsequently forms a film. The formation of acid in the liquid, due to the action of this *Pediococcus*, is very slight. LINDNER assumes that traces of lactic acid are formed. He states that in no case was he able to produce any real disease in wort or beer by inoculating these liquids with a vigorous growth of this bacterium; he concludes that the change in flavour of the beer may not be caused by this species, but by other bacteria co-existing in the infected beer.

It is supposed that Sarcina organisms, under certain conditions, are capable of producing turbidity in low-fermentation beer. F. SCHOENFELD inoculated a growth of a species which had been cultivated first in yeast-water gelatine and then on yeast-water gelatine under yeast-water, in beer pasteurised at 70° C. After some time the beer became turbid and acquired a sourish-sweet, disagreeable smell and taste. The turbidity set in most quickly when the beer was almost excluded from contact with the atmosphere.

The slime-forming species described by LINDNER has been mentioned above.

A. PETERSEN observed that an abundant development of a Sarcina could take place in bottom-fermentation lager-beer without causing any disease; on the contrary, the beer was bright and stable, and had an agreeable taste and odour.

Thus, Sarcina species exist which are not productive of any irregularity in the brewery. The same observation has been made by the author. Series of lager-beer samples have frequently been examined in the author's laboratory, all of which had the "Sarcina smell and taste," but in which, save in a few isolated cases, there was no development of Sarcina-like organisms.

A. REICHARD isolated from low-fermentation beer a Sarcina form which developed freely in unhopped wort, but not in pasteurised beer. This species developed best when the access of the air was limited. In fermentation experiments turbidity or peculiar changes of taste occurred in certain cases, but not in the majority. After many experiments he arrived at the conclusion that these contrary results were due partly to the condition of the various growths of this Sarcina form, partly also to the manner in which the fermentation took place. In cases of quiet fermentation in a larger cask the growth kept at the bottom, and the bacteria did not exert any appreciable influence on the liquid, whereas in the case of a vigorous secondary fermentation they were carried upwards in the liquid along with the carbon dioxide bubbles, after which the disease manifested itself. An addition of beer from the primary fermentation might therefore be injurious in such

cases. An addition of hops to lager-beer exerts a retarding

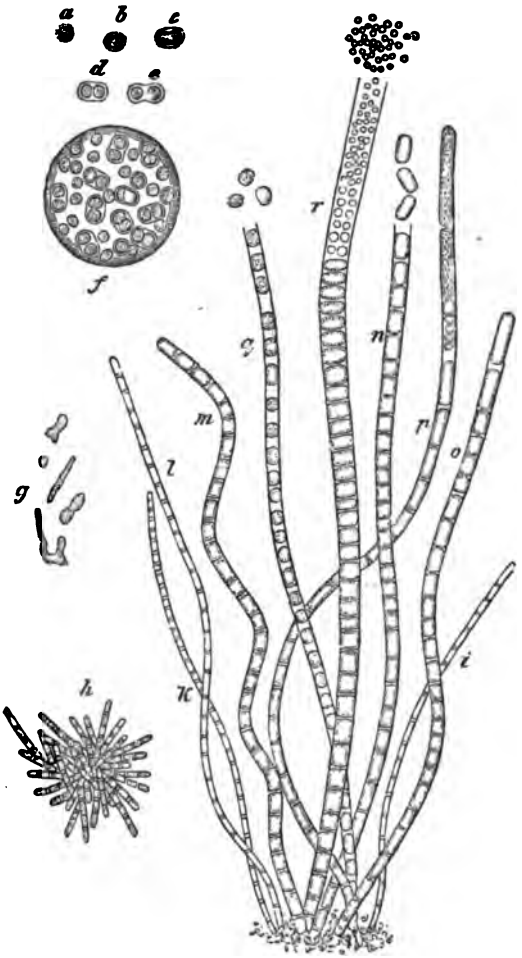

FIG. 25.—Crenothrix Kühniana (after ZOPF): *a*—*e* (600 : 1), cocci in different stages of division; *f* (600 : 1), small, round cocci-zoogloea; *g* (natural size), zoogloea; *h* (600 : 1), colony of short filaments composed of rod-like cells, originating from the germination of a small collection of cocci; *i*—*r*, filaments, partly straight, partly spirally curved (*l*, *m*), of very varying thickness, with more or less pronounced contrast between the base and apex, and different stages of division of their members and sheaths; the sheathed filament *r* shows short rods at the base, which higher up are divided into small cylindrical pieces; at the apex the cocci are seen arising from the longitudinal divisions of the cylindrical discs.

influence on these bacteria, as on the majority of bacteria occurring in beer.

Since Sarcina forms, as well as bacteria, known with certainty to be injurious, and wild yeasts occur in luxuriant growth in the *cask-deposit* it follows that, in a case of contamination arising in a brewery, special care must be taken, that the whole growth of the cask-deposit should be rendered harmless directly after emptying the casks.

In analyses of *bright wines* made in the author's laboratory, vigorous growths of Sarcina forms were frequently found; at the same time the wine had acquired a peculiar taste and odour almost like that of beers containing similar growths. However, experimental researches have not shown what part these growths play in determining the character of the wine.

9. *CRENOTHRIX.*

In microscopical examinations of water we often meet the very characteristic forms of *Crenothrix Kühniana* (Fig 25) described by COHN and ZOPF.

This ferment (frequently associated with *Beggiatoa alba*) occurs in every water which contains organic matter; sometimes it multiplies to such an extent that it may render the water unfit for use. Thus, according to ZOPF, great calamities have been caused by this fungus in the water supplies of Berlin, Lille, and certain Russian towns. In consequence of its power of storing iron compounds in its walls, it forms red or brown flocks in water. Its forms are very beautiful; it occurs in the form of cocci (*a*—*f*), which by partition and formation of viscous matter form zoogloœa; these cocci frequently grow to articulate filaments, which are provided with distinct sheaths (*h*, *i*—*r*); they increase in thickness towards their free end; when they have arrived at a certain age, they divide within the sheath into smaller pieces, which grow round and issue as rods, macro- or micrococci.

A similar species is *Cladothrix dichotoma*, which exhibits spurious ramifications and produces rod-like conidia which swarm out from the threads.

WINOGRADSKY showed that these bacteria can develop only when the nutritive medium contains ferrous carbonate. The

large deposits of iron-ochre and meadow-iron ore occurring in nature seem to be the result of the action of such bacteria.[1]

[1]In this connection may be mentioned the *sulphur bacteria* described by COHN, WARMING, ENGLER, and especially WINOGRADSKY, which occur in water, many of which produce a red-colouring matter. Under the microscope they are distinguished by the roundish bodies they contain, strongly refractive to light and composed entirely of sulphur. They are aërobic, and occur especially in waters containing sulphuretted hydrogen. This substance is oxidised by the bacteria, and the sulphur split off is stored in the cells. Among the thread-like species the *Beggiatoa alba* may be mentioned.

An important part in nature is played by bacteria which convert ammoniacal salts into *nitrates*: they are highly important for the nutrition of many plants. SCHLOESING and MUENTZ first described them; their observations were confirmed by WINOGRADSKY, who made use of pure cultures. Among these *nitrifying bacteria*, as they are termed, there are some which oxidise ammonia into nitrous acid, which is converted by other species into nitric acid. These bacteria also cause the efflorescence of nitre from walls, which often brings about the decay of brickwork, whilst snow-like masses of calcium nitrate are detached from the wall. This evil can, of course, be remedied by means of antiseptics.

CHAPTER IV.

THE MOULD-FUNGI.

MOULD-FUNGI usually affect the fermentation industries in a somewhat different manner from bacteria. Whilst the latter (regularly in distilleries, only exceptionally in breweries) make their appearance in great force during the fermentation, and are therefore able to bring about important changes in its course, and in the resulting products, mould-fungi, on the contrary, usually occur outside the true field of fermentation, selecting as their habitat the vessels, tools, rooms, the green malt, and the quiescent masses of yeast, especially top-fermentation yeast. The mould-fungi, therefore, while possessing vital importance, usually act a subordinate part. If we closely examine a growth of mould which has developed on the ceiling or walls of a fermenting room, or on the sides of a vessel, it will soon be found that we have scarcely ever to do with a mould growth *alone*; in nearly every case bacteria and yeast-like cells are found among the mould filaments. These filaments extend upwards, lifting up foreign bodies which in this exposed position are more readily carried away, partly by workmen, and partly by the air.

During malting, all sorts of micro-organisms are present on the raw materials. The mould-fungi are usually regarded as the most dangerous enemies, but this is certainly due to the fact that they are visible to the naked eye during development, and thus obtrude themselves upon our notice in an unmistakable manner. If, however, *mere numbers* are taken into account, bacteria, which are always present in

large numbers on green malt, must certainly be given the first place. It may even be considered doubtful whether the greatest influence on the product must be attributed to the mould-fungi (*Penicillium*, *Aspergillus*, etc.), when these are met with in a state of vigorous development on malt, or whether it is not far more probable that the numerous organisms accompanying them play the most important part.

The author has often found a fine white parasitic growth on the surface of pieces of pressed yeast, which most frequently consists of a mould *mycelium*, belonging principally to forms resembling *Oïdium*, *Chalara and Dematium*. It is very possible that when these plants form a thick layer on the surface of the yeast-mass, they retain by respiration a portion of the free oxygen which is necessary to enable the quiescent yeast to remain alive. Even here, without exception, bacterial growths occurred.

The fact is, that, judging from observations made in breweries and elsewhere, a growth of mould nearly always serves to indicate that other organisms of a more injurious and more active character are present in the growth. It is, therefore, of great importance that the walls of fermenting-rooms should be *smooth*; this is effected with the greatest certainty by employing the enamel paint now so much in use.

The following is a review of the most important moulds from the point of view of the fermentation industries.

1. *BOTRYTIS CINEREA* (*Sclerotinia Fuckeliana*)

forms small greyish-yellow patches on moist, decaying vegetable matter, and can also occur on wort. From the greyish-brown mycelium the conidiophores are thrown up; these are perpendicular articulated filaments, generally arranged in tufts. They grow to a height of 1 mm., after which the apical cell throws out near its point, and almost at right angles, from two to six small branches (*C'*, Fig. 26). The lowest of these branches are the longest; these again develop downwards into one or more short side branches. The topmost branches are almost as wide as they are long. Thus a system of branches

is formed which is shaped like a raceme or a bunch of grapes. When longitudinal growth is at an end, the interior of the

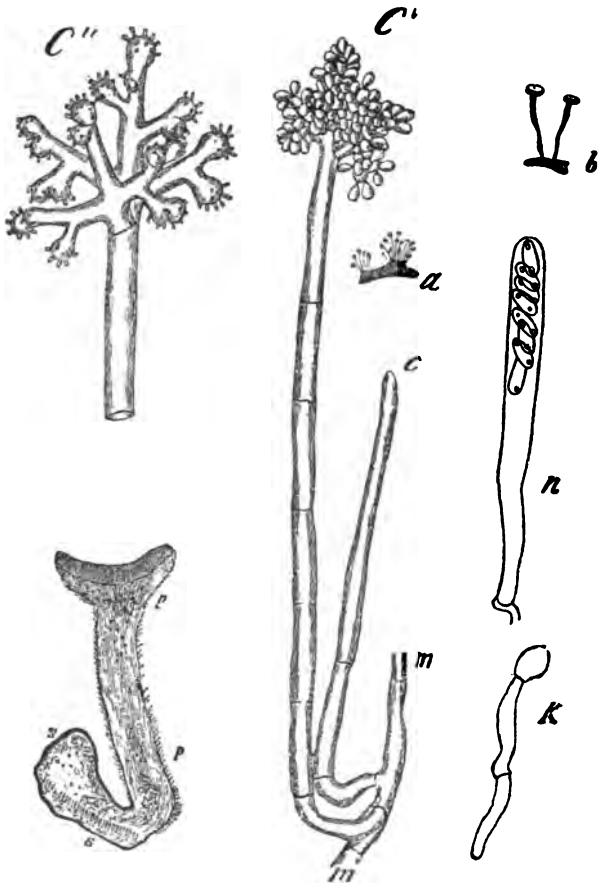

FIG. 26.—Botrytis Cinerea (after DE BARY): *a*, *b* (natural size), Sclerotia, from which at *a* the conidiophores, at *b* the apothecia (fruits with asci), are thrown out; *c*, *C*, conidiophores (*C'*, with conidia just ripe), springing from the mycelium filament *m*; *C''*, end of a conidiophore with the earliest formation of conidia from the ends of the branches; *k*, germinating conidium (×300); *p*, *s* (slightly magnified), section through a sclerotium *s*, from which a very small apothecium (*p*, *p*) is thrown up; *n*, single ascus, with eight ripe spores (×300).

branches is separated from the main stem by the formation of a transverse wall close to the latter. At the same time the ends of the branches and of the main stem swell, and on

the upper half of each swelling several small papillæ appear close together; these quickly increase to oval blisters, filled with plasma, and grow narrow, stalk-like, at their base. When these conidia (*C*) are completely developed, the walls of the branches carrying them are shrivelled up, and the conidia are consequently brought so closely together that they form a loose, irregular aggregation which readily falls off. If these clusters are placed in water, the conidia are detached from their stalks, and the envelopes of the branches, devoid of plasma, shrivel up or are only to be found in traces; their former place of attachment to the main filament appears only as a slightly raised scar. The member next below can now throw on one side the shrivelled apex, grow upwards, and form a new cluster; this can be repeated several times, whereby the conidiophores attain a considerable length. Under certain nutritive conditions the conidia and spores develop short threads from which small bright round conidia are separated, either directly or on bottle-shaped basidia.

Under certain conditions this mould can assume a peculiar *dormant state*, the so-called *sclerotium* (*skleros* = hard) (*a*, *b*, *ss*). The hyphal threads branch extremely freely, and the branches intertwine themselves into a continuous body of diverse shape, circular to a narrow spindle-shape, and of varying size; the extreme ends of the filaments are brown to black, and the ripe, solid *sclerotium* thus consists of an outer black rind and an inner colourless tissue. These bodies, which were described by DE BARY under the name of *Sclerotinia Fuckeliana*, appear as small black corpuscula occurring on the herbaceous parts of many plants, where they live as parasites or saprophytæ. They are capable, after a long period of rest—lasting at least a year—of forming a new growth. If the *sclerotium* is brought into a moist place soon after it comes to maturity, the inner colourless branches break through the black outer rind and throw up the conidiophores (*a*). If, however, the *sclerotium* is not brought into a moist place until after it has been at rest for some time, a large tuft of filaments develops from the inner tissue, and these shoot up perpendicularly and finally spread out to a flat, plate-shaped disc (*b* and *ps*); the ends of the filaments appear parallel on the free upper surface

of the disc; some of them remain thin, others swell up to club-shaped asci, and each of these asci forms in its interior eight oval spores (*n*). The mould has now entered upon the stage in which the formation of *apothecia* takes place. The spores germinate when they are set free, and the germ tubes grow into conidiophores.

According to BERSCH, FITZ, and REESS, this mould growth is the cause of one of the diseases of wine, which manifests itself as an unpleasant smoky taste and smell. Similar cases of disease have been occasionally observed in breweries; it has, however, not yet been determined with certainty whether they are caused by this mould.

In rainy seasons, when *Botrytis* attacks the grapes at a time when these are unripe, the mycelium, penetrating through the pulp, destroys the small amount of sugar in the grapes, and as it kills the cells, a fresh immigration of sugar from the leaves is checked or rendered impossible. Such grapes act injuriously upon the quality of the wine. As the mycelium penetrates into the stalks also, causing these to die off, the very young grapes on such a cluster do not generally develop, but wither away. In years when vineyards abound in good grapes, this fungus usually does not make its appearance till a short time before the close of the vintage, when it is less dangerous to the fruit; in certain countries—as, for instance, the Rheingau, Sauterne—this slow-developing grape disease, the so-called "Edelfäule," is positively liked, because the berries attacked contain a considerably smaller amount of acid than the sound ones, and the wine obtained from such grapes acquires a particularly agreeable mild taste.

2. *PENICILLIUM GLAUCUM.*

A mould which is far more widely distributed in the fermentation industries, especially in green malt, is **Penicillium glaucum**. It forms a felt-like mass on the substratum, is at first white, then greenish or bluish-grey, and spreads with great rapidity. The mycelium consists of transparent branched and divided filaments, which, when immersed in liquids, are liable to swell somewhat irregularly. From these filaments the

conidiophores (*A*, Fig. 27) are thrown up perpendicularly. They consist of elongated cylindrical cells, the terminal cell of which

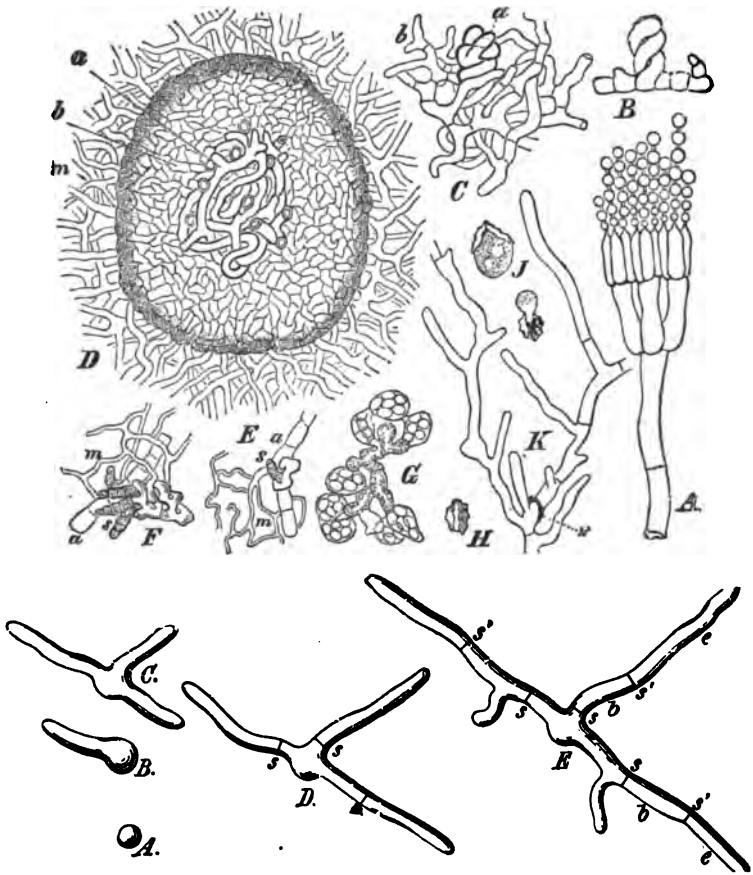

FIG. 27.—Penicillium glaucum (after BREFELD and ZOPF): *A*, conidiophore; *B*, organs of generation; *C*, first development of the sclerotium (*a*, ascus-forming hyphæ; *b*, sterile filaments); *D*, very young sclerotium in section (*a*, ascus-forming hyphæ; *b*, sterile portion of the sclerotium; *m*, mycelium); *E* and *F*, ascus-forming hyphæ; (*a*) with young asci (*s*) and sterile mycelium threads (*m*) from a more developed sclerotium; *G*, group of asci with spores; *H*, spore; *I*, germinating spores; *K*, young mycelium (with spore at *x*). *A*—*E* (below), germination of a conidium, after ZOPF (more highly magnified): *A*, conidium before germination; *B*, it has thrown out a germ tube; *C*, three germ tubes have been formed; *D*, each germ tube shows towards the spore a transverse septum (*s*); *E*, each germ tube has become divided by another septum (*s'*) into a terminal cell (*c*) and an inner cell (*b*).

soon stops in its longitudinal growth and becomes tapering and pointed; the cell next below throws out one or more opposite

branches, which rise up close to the terminal cell and, like this, consist of one pointed cell. In more vigorous individuals the branches may again ramify (compare Fig. 27*A*, upper half), or similar branches may also spring from the next cells, and these again ramify and become pointed as described above. In this tuft of branches each pointed cell (*sterigma*) breaks up into a series of spherical conidia, and finally the tuft carries a large number of conidia, arranged in series, which, when ripe, are readily scattered. These round, smooth conidia give to the patches of mould their greyish-blue colour; when they fall upon moist surfaces, they are able to germinate at once. According to CRAMER'S researches they are very resistant to higher temperatures.

In culture experiments with this fungus, BREFELD made the interesting observation that *Penicillium* can occur under certain conditions with an entirely different form of growth. He enclosed cultures of this mould-fungus on slices of coarse, non-acidified bread, between glass plates, and allowed the culture to develop while excluding air as far as possible. There then appear in pairs on the mycelium short, thick branchings, which become entwined (*B*, upper half); one part of this spiral throws out short, thick filaments (*C*), whilst the hyphal thread carrying the spiral develops numerous fine branches, which envelop the spiral and form a covering (*D*), consisting of an inner solid and an outer felt-like layer; the inner cells are gradually coloured yellow, and the outer loose cells are cast off. In this small yellow ball—*sclerotium*—a formation of swollen cells (*E*, *F*, *G*) gradually takes place by the continued branching of the above-mentioned spiral filaments, and in each of these new cells eight large and lenticular spores are produced, which have a circular furrow on the margin, and three or four slight ridges on the outer membrane (*Exosporium*). After the collapse and absorption of all the remaining elements of the interior the spores are at last set free, and the small yellow ball is then filled with the spore-dust. The entire development requires six to eight weeks. The *sclerotia* may be preserved in a dry state for several years without losing their power of germination. When the spores (*H*) are sown, the exosporium bursts open

like a shell at the circular furrow, and the endosporium swells and emerges (*I*), and elongates itself to a germ tube, which quickly develops conidiophores.

This fungus very often causes dangerous diseases in *wine*. It develops freely in casks which have not been carefully cleaned, penetrating into the wood and, in consequence of the decomposition caused by it, producing matter of a disagreeable smell and taste, which subsequently diffuses into the wine. In moist seasons it sometimes covers grapes with a luxuriant growth, giving rise to a peculiar putrefaction. The mycelium seems to penetrate not only into bruised berries, but also into those that are quite sound. These gradually change to a yellow-brown or greenish-yellow, and the fungus produces those well-known putrefaction products which are the cause of the mouldy taste of wine. The conidia of this fungus are also able to germinate in the juice of grapes, on which the mycelium is supposed to exercise a prejudicial influence.

Penicillium possesses the power of secreting an *invertive ferment* which is able to convert cane-sugar into other sugars; it also contains *diastase* and *maltase* (BOURQUELOT). This fungus is made use of in the manufacture of Roquefort cheese.

WEHMER has described two fungi, *Citromyces Pfefferianus* and *C. glaber*, which have nearly the same construction as Penicillium. These form a green covering on suitable nutritive media, in which, given a sufficient supply of air and a suitable temperature, they convert the sugar present into *citric acid*. It is worthy of note that these two fungi are almost insensible to the citric acid accumulating in the nutritive liquid; indeed, they remain unaffected by even comparatively high concentrations, whilst other acids, and specially mineral acids, even in small quantities, are apt to check their growth. Both of them, according to WEHMER'S statement, are made use of technically in the preparation of this acid.

A mucor species (M. pyriformis) has also been utilised in this process.

3. *EUROTIUM ASPERGILLUS GLAUCUS.*

The development of this fungus was first thoroughly described by the celebrated DE BARY. It forms a fine felty, greyish or greyish-green covering on various materials, and is able to grow with the greatest luxuriance on green malt.

The mycelium consists, as in the case of *Penicillium*, of fine transparent and branched threads, provided with transverse septa. Some of the hyphal threads are thrown up perpendicularly, are thicker than the rest, and very rarely branched or divided by septa. Their upper ends swell to spherical flask-shaped heads (*c*), and these throw out from their entire upper portion radially divergent *papillæ* of an oblong form; these *sterigmata* then throw out at their apex small round protuberances, which are attached to the *sterigmata* by greatly constricted bases, and after some time are defined from the former as independent cells (spores, or *conidia*). Below the base of the first spore, a second begins to form from the crown of the *sterigma*, and pushes the first upwards; a third then forms, and so on. Each *sterigma* thus carries a chain of spores, the youngest of which is closest to the *sterigma*. This occurs at the same time over the whole surface of the enlarged ends of the conidiophore, which is thus finally covered with a thick head of radially-arranged chains of spores. These masses of spores form the greyish-green dust which covers the mycelium.

Finally, the *conidia* separate from one another; they have then a warty appearance on their outer surface. These small bodies are able to germinate (*p*) directly after they have become detached, and quickly develop a new mould-fungus; on this fact depends the rapidity with which the plant spreads. Under certain conditions, which are not yet sufficiently known, but which in every case appear to be connected with a free supply of nutriment, the mould develops *perithecia*. These appear at first as tender branches, which, at the termination of their longitudinal growth, begin to twine their free ends in the form of a spiral of four to six turns (*f*); the threads of the spiral gradually approach nearer together, until finally they are brought into contact, so that the entire end of the

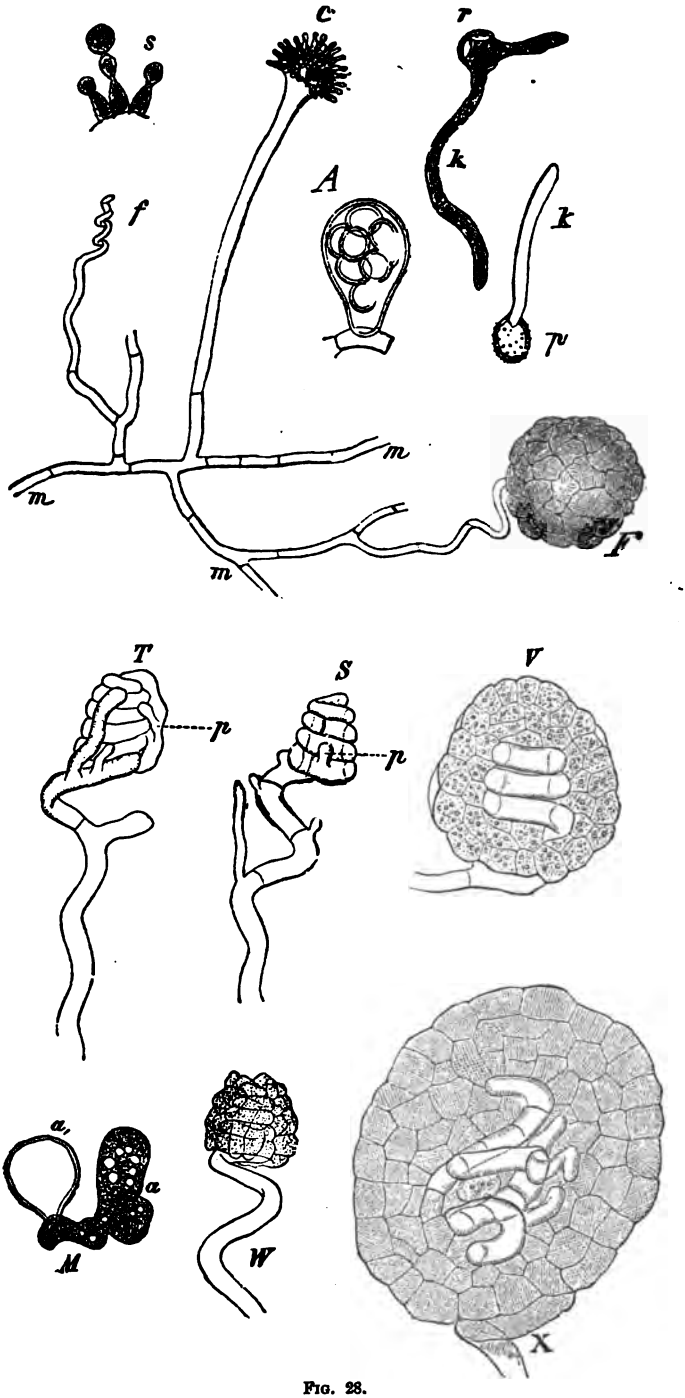

Fig. 28.

filament takes the form of a helix (the *ascogonium*). There then grow from the lowest turn of the helix two or more small branches, which cling closely to the spiral. One of these (*S*, *T*, *p*) quickly outstrips the others in growth; its upper extremity reaches the uppermost turn of the helix, and fuses with it. The other branch or branches likewise grow upwards along the spirals, shoot out into new branches, and gradually become so interlaced that the spiral is finally surrounded by an unbroken envelope (*W*). These branches divide slowly into septa perpendicular to the surface, and the envelope consequently consists of short, angular cells, in which new septa appear parallel to the surface, so that the envelope thickens and is composed of many layers (*V*, *X*, *F*). The small sphere now formed is about one-quarter mm. in diameter; the outermost layer is yellow, whilst the inner layers remain soft, and later are dissolved. The spiral after a time extends and throws out on all sides branched filaments, which dislodge the inner layers of the envelope. These branches finally take the form of an *ascus* (*M* and *A*), eight spores being formed in each. After the breaking up of the *asci* the spores lie loose in the interior of the *perithecium*, and are liberated by the rupture of the fragile wall of the latter. The spores, as in the case of *Penicillium*, are bi-convex, warty, and possess a stout outer membrane and an inner one, which, on germination, bursts the outer membrane forming two valves (*r*).

Eurotium Aspergillus glaucus contains a *diastatic* ferment, which converts starch into dextrin and maltose.

In addition to this species, several others, closely related, occur in nature, and also find their way into the factory.

Thus, according to COHN, it is *Aspergillus fumigatus* that

FIG. 28.—Eurotium Aspergillus Glaucus (DE BARY): *m*, *m*, hyphal thread, carrying a conidiophore *c* (from which the conidia have fallen), a perithecium *F*, and the first rudiments of an ascogonium, *f* (×190); *s*, three sterigmata from the crown of a conidiophore, showing the conidia-constrictions; *p*, germinating conidium (×250—300); *A*, Ascus; *r*, germinating ascopore; *k*, germ tubes; *S*, spiral ascogonium; at *p* the commencement of the growth of one of the enveloping hyphæ; *T*, older stage; *W*, ascogonium, already surrounded by the envelope; *V*, longitudinal section of an older stage; in the centre the ascogonium, surrounded by the envelope, which now consists of several layers; *X*, longitudinal section of a later stage of development; the ascogonium is enveloped in a sheath of many layers; it has loosened its convolutions, and is beginning to throw out the ascus-forming branches; *M*, portion of an older ascus-bearing branch; *a*, a young ascus; a_1, an older ascus which has burst.

causes the temperature of germinating barley, in badly managed heaps, to rise to 60° C. or even further.

Aspergillus (*Sterigmatocystis*) *niger*, according to investigations made by GAYON and BOURQUELOT contains a number of different ferments; for example, diastase, invertase, maltase, and emulsin.

In the greater number only the conidia stage is known.

4. *ASPERGILLUS ORYZÆ.*

In the preparation of the strongly fermented *Japanese rice wine* ("saké"), *Aspergillus Oryzæ* is systematically employed. Rice grains, freed from their hulls, are steamed, but the aggregation and gelatinisation of the grains are avoided. In order to prepare a malt serviceable for the brewer from these grains, which are not capable of germination, and from which the ordinary diastatic action is consequently excluded, the mass of grain is mixed with the so-called "Tane kosi"—rice grains, which are coated over with the mycelium and conidia of *Aspergillus Oryzæ*; or the yellowish-brown spores of the fungus are mixed with the steamed rice grains. In moist and warm air there develops on the rice at the end of about three days a white velvety mycelium, which gives to the whole mass an agreeable odour, resembling apples or pine-apples. Before the fructification of the fungus takes place, a fresh quantity of steamed rice is introduced, and this also is gradually coated over with mycelium; this process is repeated several times. In the "koji" mass thus produced a part of the starch has been hydrolised, and some of the albuminoids have been rendered soluble. The koji-mass is mashed, 21 parts of koji being mixed with 68 parts of steamed rice and 72 parts of water. This pasty mass ("moto") is allowed to remain at about 20° C.; after some days it clarifies, the conversion of starch and dextrin into sugars progresses, and at the same time a spontaneous and very violent fermentation sets in. In this fermentation there occurs a Saccharomyces which is able to produce a very high percentage of alcohol. The mass is now heated up to about 30° C. At the end of two or three weeks the primary fermentation is finished. The product, after being

filtered, is subjected to a secondary fermentation, and the liquid is then clear, yellow and sherry-like, containing 13 to 14 per cent. of alcohol. It is then commonly pasteurised at 44° C. in iron vessels.

Aspergillus Oryzæ, according to KELLNER, also plays an important part in the preparation of *Chinese Shoyn or Soy*.

The raw material is a mixture of soy beans, wheat, common salt, and water. Part of the wheat is ground, steamed, and mixed with koji, whilst the rest of the wheat is parched and ground. Then both portions are mixed with the ground and boiled beans, and the koji-fungi now cover the whole mass; the latter, after a lapse of some days, is mixed in large tuns with salt and water, and then left to stand for a fermentation, which often lasts several years. The liquid is then expressed from the grains.

ATKINSON has found a ferment in koji which is soluble in water, inverts cane-sugar and converts maltose, dextrin, and starch-paste into dextrose. The researches of KELLNER, MORI, and NAGAOKA have likewise shown that the koji-mass possesses a strongly invertive ferment, which converts cane-sugar into dextrose and levulose, maltose into dextrose, and starch into dextrin, maltose, and dextrose. The various micro-organisms which occur in the koji-mass in all likelihood possess different invertive ferments.

In Java, the *Aspergillus Wentii*, described by WEHMER, is used for the preparation of *soy* and the so-called "*Taotjiung*" (bean's pulp). It occurs spontaneously on soy beans. This species forms a snow-white mycelium, which is later coloured brown by the globular and usually feebly warty conidia; the sterigma are not ramified. According to PRINSEN GEERLIGS, who described the technical use of the fungus, it not only possesses a peptonising and diastatic ferment, but is also able to partially dissolve the cell-walls of the soy beans. According to WENT, this fungus also develops perithecia.

5. *MUCOR*.

The genus *Mucor* belongs to the most interesting of the groups of mould-fungi with which we have to deal, since it

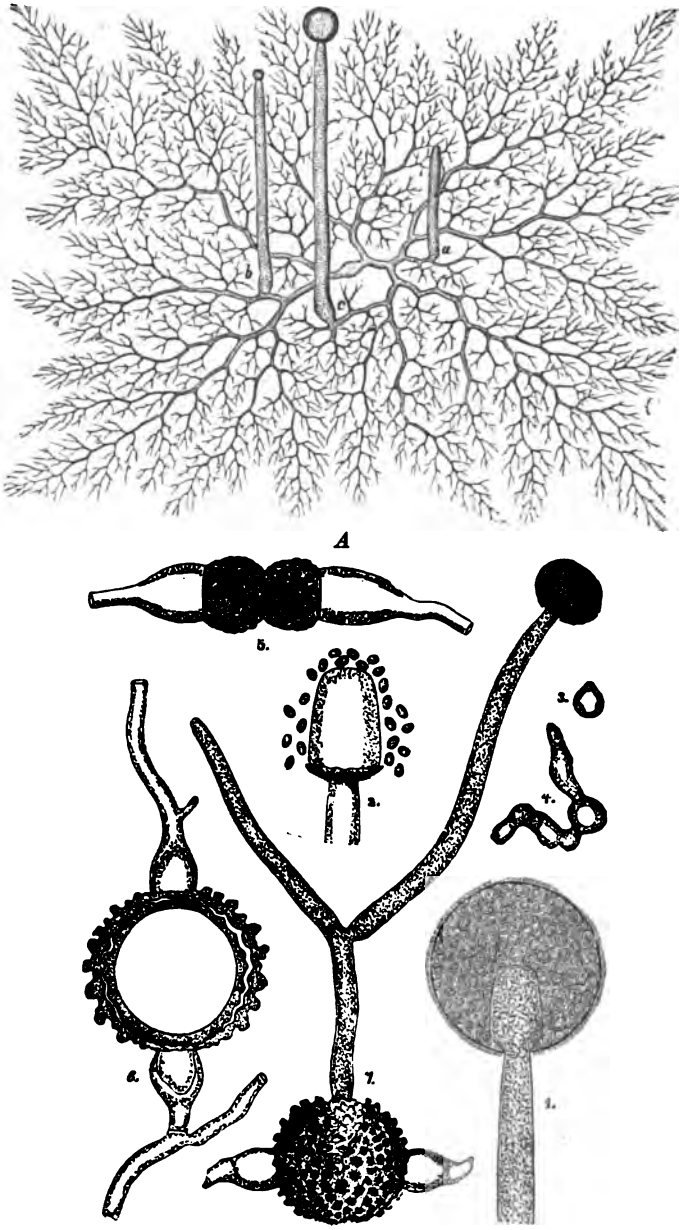

FIG. 29.—Mucor Mucedo (after BREFELD and KNY): *A*, tree-like ramified mycelium with isolated thicker upright branches (*a*, *b*, *c*). 1, Sporangium; 2, columella and spores; 3, 4, germinating spores; 5, 6, development of the zygospore; 7, germinating zygospore with sporangium.

embraces species with very marked fermentative action. These generally occur as a grey or brown felt-like mass, often attaining a considerable height—occasionally measuring several inches—in which small yellow, brown, or black spherules can be distinguished by the naked eye.

We shall describe the most frequently occurring species.

Mucor Mucedo (Fig. 29), one of the most beautiful mould-fungi, and one which occurs very generally on the excreta of phytophagous animals, has a transparent white mycelium, which develops numerous and delicate ramifications on the surface of and within the substratum, and which, in its earliest stages of development, and until the sporangia begin to form, is without transverse septa, and therefore unicellular. From the mycelium are thrown up single vigorous branches, the sporangium-carriers; the points of these branches which, according to ZOPF, contain a reddish-yellow fatty colouring matter, swell greatly, and below the swelling a transverse septum is finally formed, whereby the sporangium is marked off from the sporangium-carrier. The transverse wall arches upwards, and forms a short column—termed the *columella* —in the interior of the spherical head, whereby an inner space of peculiar form (1) results. The protoplasm of this space breaks up into a number of small portions, which are gradually surrounded by a membrane and rounded off; these are the spores. At the same time the sporangium is coated on its outer surface with small needle-shaped crystals of calcium oxalate. As soon as the ripe black sporangium takes up moisture, the wall is dissolved, and the spores with their yellowish contents are scattered on all sides along with the swelling contents of the sporangium. The columella, which projected upwards in the sporangium, still remains at the end of the sporangium-carrier; this is now surrounded at its base by a collar (2), the remains of the outer wall of the sporangium. When the refractive spores fall on a favourable substratum, they swell very considerably and send out one or two germ tubes (3, 4), which quickly develop to a vigorous mycelium.[1]

In addition to this mode of reproduction, *Mucor Mucedo* and

[1] Many of the botanical characters stated above do not apply to M. Mucedo alone, but must rather be considered as generic characters.

the other species possess also a sexual method of reproduction, which takes place by means of a conjugation of two branches of the same mycelium. Two such short branches, filled with plasma, and growing towards each other, form club-like swellings and come in contact at their free ends, which flatten out (5). Each of the branches is then divided into two cells by a septum, and the end cells, which are in contact (the conjugating cells), coalesce by dissolution of the original double wall which separated them. The two conjugated cells are either equal in size, as in *Mucor Mucedo*, or unequal, as in *Mucor stolonifer*. The new cell thus formed—*zygospore* (6)—quickly increases in size and expands to the shape of a ball (in *Mucor stolonifer* to the shape of a barrel), after which the wall thickens and forms stratifications; externally it is coloured dark and covered with wart-like excrescences. These outer layers are very resistant to the action of acids. The contents possess an abundance of reserve substance (fat). The zygospores are generally able to germinate only after a long period of rest; the germ tube, after bursting the outer layers, quickly develops the sporangia mentioned above (7). In the zygospore we thus find a *resting-stage* of the plant, an organ which by its structure enables the mould to preserve life during periods which are unfavourable for growth.

The morphological characteristics given above are, for the most part, noticeable in the following species.

Mucor racemosus, which occurs on bread and decaying vegetable matter in very variable forms, has a branched, multicellular sporangium-carrier, which can also attain to a considerable height. The brownish sporangia are developed at the ends of the branches. The spores are colourless. When this fungus is cultivated in wort, the submerged mycelium swells irregularly, and a large number of transverse septa appear, which divide it into large barrel-shaped or irregular cells filled with highly refractive plasma. These cells—*gemmæ*—are readily separated, and then assume a spherical shape (compare Fig. 30, 7), as was first observed by BAIL, and *multiply by budding like the true yeast-fungi; the same takes place with the submerged spores* (*Mucor-yeast, spherical yeast*). If the gemmæ are carried to the surface of the liquid, they are

again able to develop the mould-form. The mycelium produces a similar characteristic formation of gemmæ when cultivated on solid substrata. The plasma of the filaments collects in certain places in a compact mass, and is then enclosed at both ends by a transverse wall. At the same time the cell swells, the walls grow thicker, and fatty substances are stored

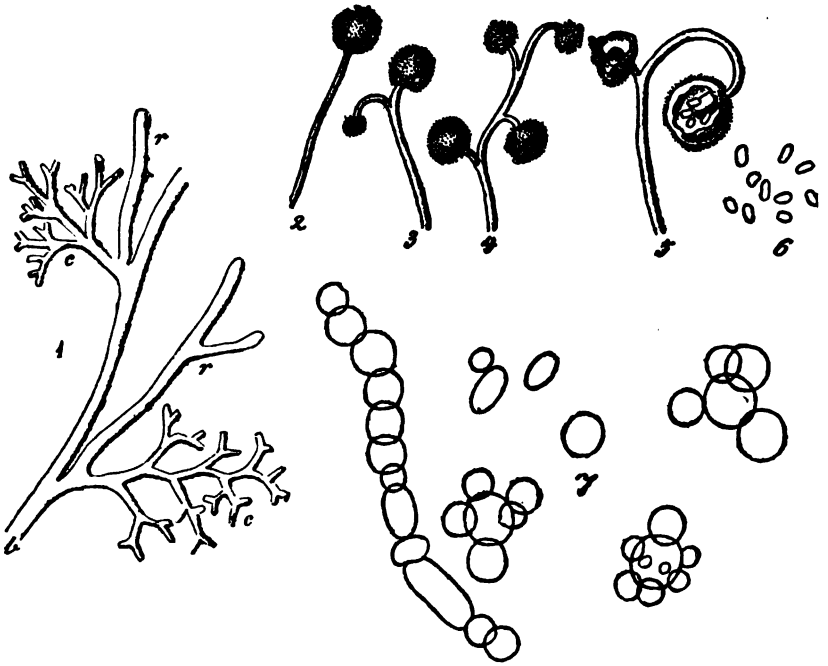

FIG. 30.—Mucor Circinelloides (after VAN TIEGHEM and GAYON): 1, Mycelium; *b*, main branch; *c*, root-like branches; *r*, axillary branches; 2—4, development of sporangia; 5, opened sporangia; 6, spores; 7, submerged mycelium and budding cells.

in the interior. The intermediate portions of the hyphæ gradually lose their contents.

Mucor erectus, occurring, for instance, on decaying potatoes, has the same microscopic appearance as *Mucor racemosus*; physiologically, however, it differs from this species.

Mucor circinelloides (Fig. 30) has a very characteristic appearance. The mycelium (1) shows the remarkable branching which occurs in some of the species of *Mucor*:—the

main branches (*b*) send out short, root-like, branches with frequent forking (*c*); at the base of these come new mycelial branches (*r*), which grow erect, and are able to form sporangia (2 to 5); the sporangium-carrier is branched. During its development it becomes considerably curved, and to this the species owes its name of *circinelloides*. In this form, as in *Mucor spinosus*, whose chocolate-brown sporangia are distinguished by the columella being studded on its uppermost part with pointed, thorn-like protuberances, the mycelium, when submerged in a saccharine liquid, produces gemmæ, similar in formation to those of *Mucor racemosus* and *Mucor erectus*.

Mucor stolonifer (*Rhizopus nigricans*) attains a considerable size, and occurs very commonly, for instance, on succulent fruits. This mould is easily recognised, for its brownish-yellow mycelium sends out, diagonally, thick hyphæ without septa. These attain a length of about 1 cm., then, sinking their points to the surface of the substratum, send out fine ramified hyphæ, resembling rootlets, into the latter, whilst other hyphæ rise perpendicularly and develop sporangia; other branches again form new "runners." The black sporangium possesses a high, dome-shaped columella, and develops a number of dark-brown spores, round or angular. When these are freed by the absorption of the sporangium wall, the columella curves over on the sporangium-carrier like an umbrella, the line of junction of the external wall remaining in evidence in the form of a collar. In this species no formation of gemmæ has been observed.

The species of *Mucor* have very considerable interest from one point of view, since they are able to act, in different degrees, as true *alcoholic ferments*. Their fermentative power is not related exclusively to their power of forming budding gemmæ, since these have not been observed in *Mucor Mucedo* and *stolonifer*.

According to HANSEN'S investigations, the various species, as far as they really are alcoholic ferments, induce fermentation not only in solutions of dextrose and invert-sugar, but also in solutions of maltose. Of all the species which he investigated, *Mucor racemosus is the only one capable of inverting*

a cane-sugar solution; the others are consequently unable to bring about fermentation in a solution of this sugar.

The most active fermentative power is possessed by *Mucor erectus*. In beer-wort of ordinary concentration—14° to 15° Balling—it yields up to 8 per cent. by volume of alcohol. It also induces alcoholic fermentation in dextrin solutions, and converts starch into reducing sugar. *Mucor spinosus* yields up to 5·5 per cent. by volume of alcohol in beer-wort. In maltose solutions distinct fermentation phenomena were observed, and after the lapse of eight months the liquid contained 3·4 per cent. by volume of alcohol. *Mucor Mucedo* has only a comparatively feeble fermentative power both in wort (up to 3 per cent. by volume of alcohol) and in maltose and dextrose solutions. *Mucor racemosus* produces in wort as much as 7 per cent. by volume of alcohol, develops invertase, and ferments the inverted cane-sugar; thus, as mentioned above, it occupies a unique position.

Mucor circinelloides, according to GAYON, is without action on cane-sugar, whilst it exercises a very powerful action on invert-sugar (yielding 5·5 per cent. by volume of alcohol). GAYON concluded that this mould might with advantage be employed to extract cane-sugar from molasses in the manufacture of sugar. However, so far as the author has been able to learn, this observation has not yet received any practical application.

Another fungus belonging to this group is *Mucor Amylomyces Rouxii* described by CALMETTE and EIJKMAN, which occurs in so-called "Chinese yeast" in the form of small white-grey cakes, consisting of rice-corns kneaded together with different sorts of spice. These cakes are pulverised and mixed with boiled rice, which is soon covered with a web of the mould's white mycelium; by slow degrees the rice is converted into a yellowish liquid, which contains glucose, produced by the vigorous diastatic ferment of the fungus. The latter, like the other Mucor species, also possesses an alcoholic ferment.

The *Chlamydomucor Oryzæ* described by WENT and PRINSEN GEERLIGS, which may be identical with the previous species, contains similar diastatic ferments. It is used in Java in the fermentation of arrack.

6. *MONILIA.*

A large number of different fungi of comparatively simple structure are described under this name in works on mycology. From a mycelium, the colour of which varies according to the species, branches are thrown up, which gives rise to series of egg-shaped or elliptical spores. The genus has attracted interest on account of one of its species, provisionally named *Monilia*

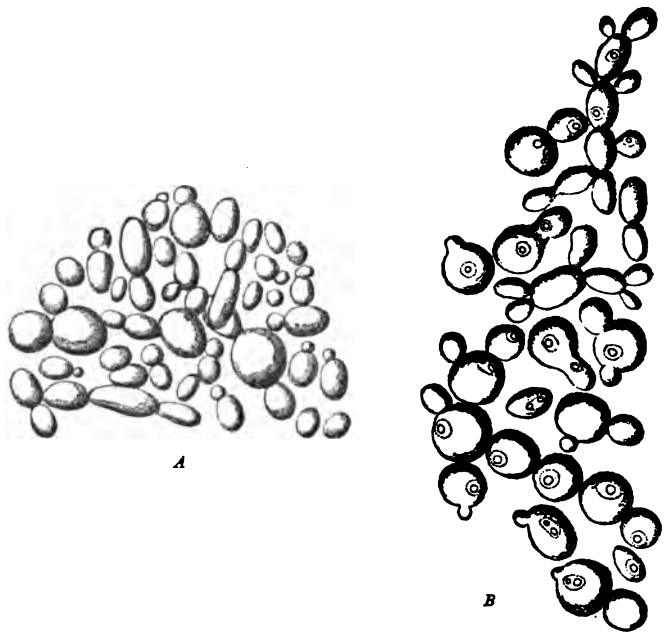

FIG. 31.—Monilia candida (after HANSEN): *A*, growth in beer-wort or other saccharine nutritive liquids; *B*, cells of a young film-formation.

candida by HANSEN from BONORDEN'S description, which shows very remarkable physiological properties. It occurs in nature in the form of a white layer covering fresh cow-dung, and on sweet, succulent fruits. When introduced into wort, it develops a copious growth of yeast-like cells, which resemble *Saccharomyces ellipsoideus*, or *cerevisiæ*. At the same time it excites a vigorous alcoholic fermentation, and whilst this is progressing forms a Mycoderma-like film on the liquid;

the cells in this film extend further and further, and finally form a complete mycelium. During the early fermentation the fungus produced only 1·1 per cent. by volume of alcohol, whilst *Sacch. cerevisiæ* gave 6 per cent.; but the *Monilia* continued the fermentation, and produced, at the end of six months, 5 per cent. by volume of alcohol, whilst the culture-yeast did not give more than the quantity quoted above.

According to HANSEN, *Monilia* does not secrete *invertine*, but nevertheless ferments *cane-sugar*, from which he concludes that the cane-sugar is directly fermentable; however, he indicates the possibility that cane-sugar is converted into invert-sugar in the interior of the cells, and that this latter form of sugar is immediately utilised.

FISCHER and LINDNER confirmed this by chemical analyses; they showed that neither from the fresh nor from the dried growth can any substance be extracted capable of hydrolising cane-sugar. On the other hand, they were able to invert cane-sugar by using the dried fungus itself, in presence of anæsthetics, and also by using a fresh growth, part of the cells being disrupted by grinding with glass-powder. The authors infer from this that an inverting enzyme, which is insoluble in water, forms a part of the living plasma, and that consequently the fermentation of cane-sugar is preceded by inversion, even in the case of Monilia. It has not yet proved possible to isolate the ferment.

Maltose, according to FISCHER, is split up both by fresh and by dried Monilia, and also by an aqueous extract of a dried growth; he therefore infers that Monilia contains the enzyme *maltase* recently discovered by him in Saccharomyces cerevisiæ.

According to BAU, Monilia also ferments *dextrin* generated by diastase.

Up to a recent date (1883), Monilia candida was the only fungus which was known to be capable of fermenting cane-sugar, although not secreting invertine. Since then ZOPF, BEIJERINCK, BEHRENS, and other investigators have discovered a few micro-organisms which possess this property, but the phenomena must still be considered exceptional. It has

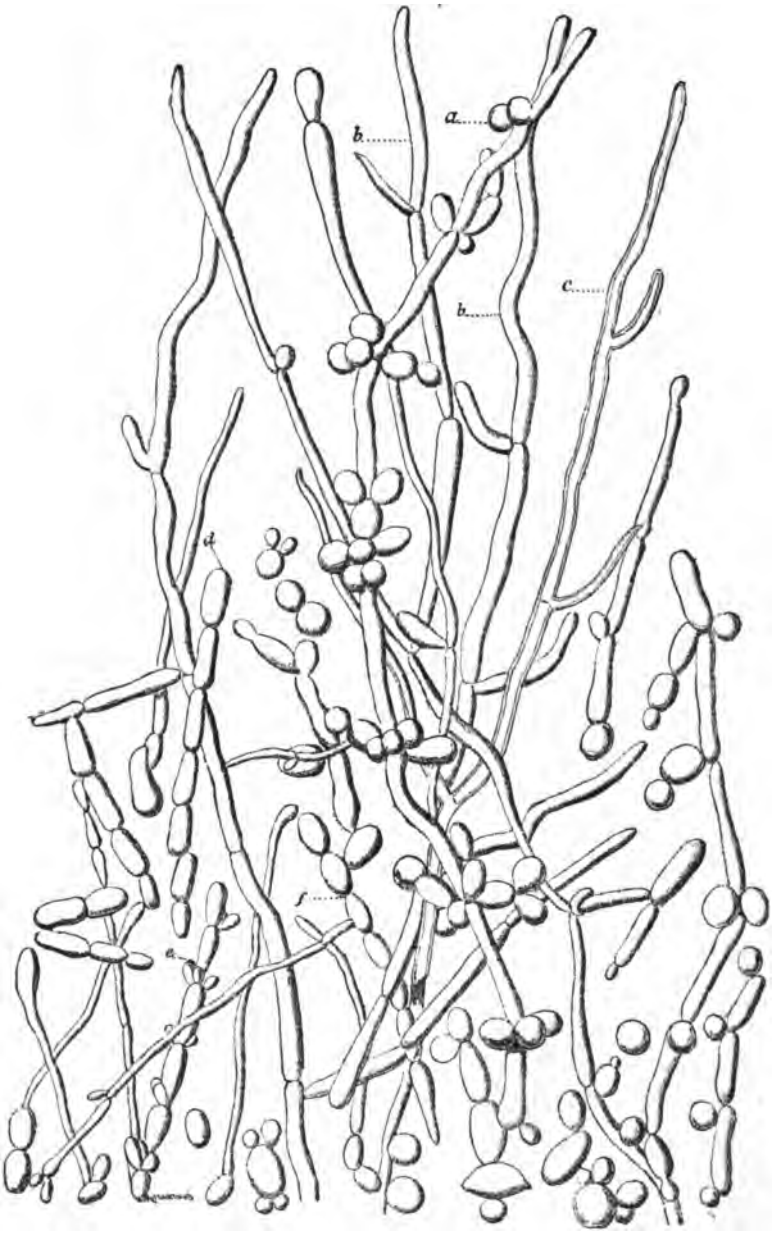

FIG. 82.

the greatest interest as an indication of the existence of unexpected gradations even in this natural domain.

A certain amount of carbon dioxide and ethyl alcohol is developed in liquids undergoing a Monilia fermentation.

Finally, it is worthy of mention that this fungus is distinguished by its power of withstanding high temperatures. In beer-wort and cane-sugar solutions it develops vigorously at 40° C., and induces an active fermentation at this temperature.

7. *OÏDIUM LACTIS.*

A mould-fungus which has played an important part in the literature of the physiology of fermentation and in that of medicine is *Oïdium lactis*, the so-called lactic acid yeast.

Some authors have sought to establish the theory that this fungus is a stage in the development of species which, under other circumstances, occur in entirely other forms, and with quite different properties. It was thus brought into genetic relation with Bacteria, Chalara (see below), Saccharomyces, etc. Both BREFELD and HANSEN have carried out numerous investigations with this fungus, and have undertaken culture experiments, which were continued for a long time without producing any other than the ordinary Oïdium-form. Recently, it is true, BREFELD has discovered, in several higher fungi, a formation of conidia resembling chains of Oïdium cells; but it has not yet been determined whether this also includes that particular species which we designate *Oïdium lactis*.

FRESENIUS correctly gave to this species the specific name of *lactis* (of milk); for universal experience goes to show that it has its ordinary habitat in milk, where it can be found, in the

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FIG. 32.—Monilia candida (after HANSEN): growth of mould; forms like *a* are frequent; they consist of chains of elongated cells, more or less thread-like, and rather loosely united; at each joint there is generally a verticil of oval cells, which readily fall off; *b* represents another form, also of frequent occurrence, but distinguished from the former by having no verticillate cells; instead of these there generally issues from every joint a branch of the same form as the mother cell, but shorter; the links of these chains are often closely united, the constrictions in many cases disappear, and a very typical mycelium, with distinct transverse septa (*c*) is produced; the forms *b* and *c* occur in the nutritive medium, *a* commonly on the surface. Forms like *d* have much resemblance to Oïdium lactis. *e* shows a chain of pear-shaped cells with verticils of yeast-cells resembling Saccb. exiguus; the chain of lemon-shaped cells represented at *f* closely resembles Ehrenberg's figures of Oïdium fructigenum. Between the principal forms described there are numerous yeast-cells of different forms, variously arranged in colonies; as is usually the case, there also appear forms like Saccb. conglomeratus Reess.

majority of cases. However, as yet no evidence has been brought forward that this mould-fungus stands in causal relation to the acid fermentations of milk. Further, it occurs spontaneously in various other liquids, and among these in the saccharine mixtures which are employed in the fermentation industries, and in these it is able to induce a feeble alcoholic fermentation. Thus, according to LANG and FREUDENREICH, it produces in milk and grape-sugar solutions, in the course of about 10 days, 0·55 per cent., in 5 weeks, 1 per cent. by volume of alcohol; smaller proportions of alcohol are produced in cane-sugar and maltose solutions. The same authors found that the fungus possesses the faculty of decomposing albuminoid matter to a high degree. Cultures made in milk-sugar nutritive solution develop a powerful odour resembling that of soft cheese (Limburg); the Oïdium doubtless has something to do with the ripening of this sort of cheese.

The hyphæ, which are often forked, branched, thin-walled and transparent (Fig. 33, 1), form a thick white felt; in the upper part of the filaments transverse septa are formed close together, after which the single cells, filled with very refractive plasma, are detached as conidia (Fig. 33, 3 to 7, 11 to 14, 17 to 19). When the fungus grows on solid substrata, the hyphæ unite and form remarkable conical bodies. As a rule, the conidia, in longitudinal section, are rectangular with rounded corners (Fig. 33, 3, 6, 17 to 19); in a growth of this mould-fungus, spherical, roundish, pear-shaped, and quite irregular conidia (Fig. 33, 4, 5, 11 to 14) are, however, almost always present. These organs of multiplication, the only ones known, send out one or more germ-tubes.

The fungus may occur in beer, especially when poor in alcohol. As the amount of alcohol increases, the conditions for its growth become more unfavourable; still, neither wort nor beer is exposed to the danger of being attacked to any extent by *Oïdium*, since it is not able to compete in the struggle for existence with the concourse of organisms which at once appear when fermentable liquids are exposed to the atmospheric germs.

In numerous investigations with top-fermentation yeast, the author has found that it offers a very favourable nutritive material for this fungus, especially when the yeast is in a quiescent

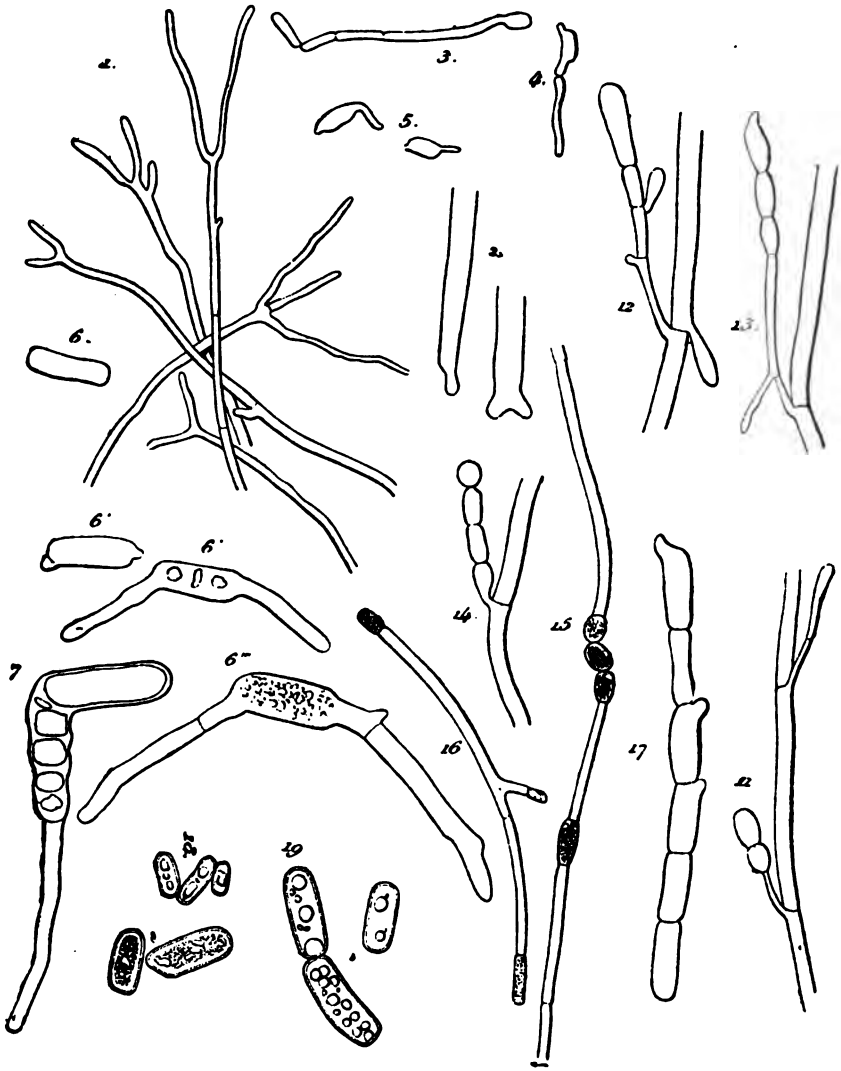

FIG. 33.—Oïdium lactis (after HANSEN): 1, Hyphæ with forked partitions; 2, two ends of hyphæ—one with forked partition, the other with commencement of development of a spherical link; 3—7, germinating conidia; 6—6‴, germination of a conidium, sown in hopped beer-wort in Ranvier's chamber, and represented at several stages; at each end germ tubes have developed; after 9 hours (6‴) these have formed transverse septa and the first indications of branchings; 11—14, abnormal forms; 15, 16, hyphæ with interstitial cells, filled with plasma; 17, chain of germinating conidia; 18, conidia which have lain for some time in a sugar-solution; the contents show globules of oil; 19, old conidia.

state at the end of the fermentation. Sometimes a microscopic examination has shown an enormous number of conidia. It is not known what influence such a growth exercises on the quality of the yeast and the beer, but without doubt it is advisable to avoid the fungus as much as possible.

8. The red colour occasionally occurring on malt-corns is due to various fungi, among which is a *Fusarium* described by

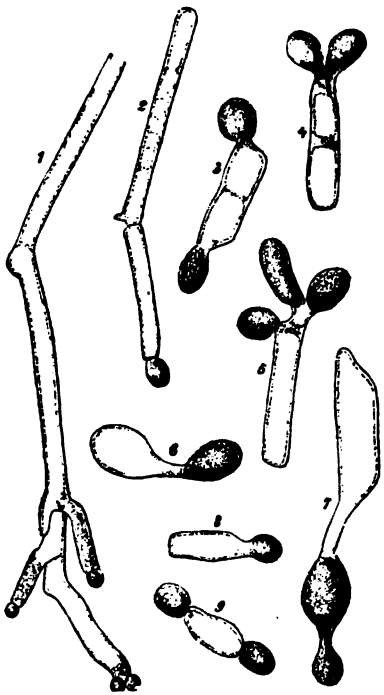

FIG. 34.—Chalara Mycoderma (after HANSEN): 1, a branched hypha, the terminal limb of which is throwing off conidia; 2, a hypha, at the upper cell of which a sterigma, which has thrown off conidia; 3—9, various forms of links of hyphæ, which are separating conidia.

MATTHEWS and KLEIN. The mould-formation begins on the germinating part of the corn, and from thence spreads over its surface. The filaments of the mycelium, which show globular swellings, are connected by numerous bridges, as it were. The red colouring matter is located in the contents of the filaments. On a moist medium the membranes gradually swell,

forming a slimy envelope which is coloured violet by iodine. The oval conidia germinate either directly or previously grow into sickle-shaped cells consisting of several links. Germinating filaments issue from the points of the latter, and by slow degrees the cells swell up. Both the mycelium and the sickle-shaped conidia are able to produce thick-walled spores. The fungus does not appear capable of hindering the growth of sound malt-corns, even if its mycelium spreads freely over their surface. Generally speaking, it only attacks diseased corns.

9. *Chalara Mycoderma* (Fig. 34) is described in PASTEUR'S "Études sur la bière" as one of the organisms commonly occurring on the surface of grapes. The mycelium forms a film on liquids, and consists of branched, greyish filaments, often filled with highly refractive plasma, which develop at different points conidia of unequal form and size. CIENKOWSKI, in his memoir on the fungi occurring as films, first gave a detailed description of *Chalara*. HANSEN found that this mould-fungus develops in both ordinary and diluted wort and lager beer.

10. A mould-fungus about which a great deal has been written in the literature of our subject is *Dematium pullulans* (Fig. 35), which was first described by DE BARY, and more minutely by LOEW. It frequently occurs on fruits, especially grapes, and has a branched mycelium, from which buds are thrown out; these have a striking resemblance to ordinary yeast-cells (4), and are able either to propagate through many generations by yeast-like budding, or to produce germinating threads, which give rise to a mycelium (3). When this has attained a certain age, it forms numerous closely situated transverse septa, and gradually becomes brownish or olive-green (5); in this we have the resting stage of the plant. In a species bearing morphologically a close resemblance to Dematium pullulans a growth of *endogenous spores* in the filaments was observed by F. WELEMINSKY in the author's laboratory. In HANSEN'S air-analyses *Dematium* was very frequently found, from spring until late autumn, in wort to which the air had access; he observed that when the mould was sown in a saccharine liquid, it at first only developed mycelial threads; after some time, however, the yeast-like cells were separated, without inducing alcoholic fermentation.

Quite recently the author, also JUST CHR. HOLM, JOHAN-OLSEN, and others, have found Dematium species with endogenous spore-formation; from these spores develop bud-

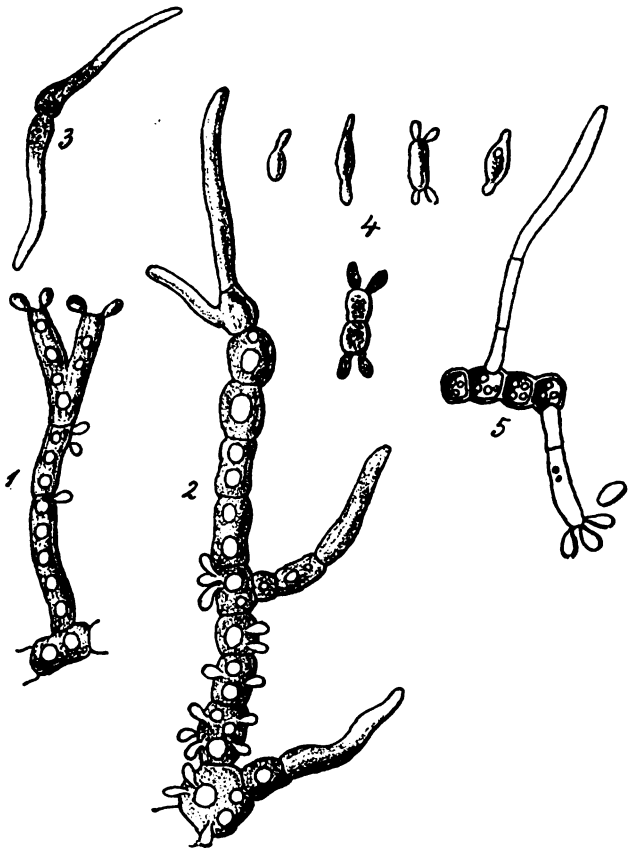

FIG. 35.—Dematium pullulans (after LOEW): 1, 2, full-grown mycelial threads with yeast-like cells; 3, cells of the latter kind developing to mycelial threads; 4, cells with yeast-like buds; 5, appearance of yeast-like cells on the germ tubes of the brown-walled cells.

ding fungi, also producing endogenous spores, and consequently belonging to the Saccharomycetes.

H. STOECKLIN found in the filaments of *Oïdium albicans* (Saccharomyces albic.) a similar endogenous spore-formation; budding fungi were developed from the spores.

11. *Cladosporium herbarum* is a mould which may occur in fermentable liquids and in fermenting rooms. This organism sometimes occurs in very large quantities in the latter; some years ago the author found the ceiling and a portion of the walls in a bottom-fermentation room thickly covered with small black patches; these consisted of Cladosporium, whose conidia were consequently always found in the yeast. The plant consists of a yellowish-brown mycelium, with short, straight filaments, stiff and brittle: those growing erect can produce at their upper extremities conidia of very varying forms—spherical, oval, cylindrical, straight, or curved. The systematic position of the mould and its possible genetic connection with other known fungi is just as little established as its influence on nutritive liquids. ERIKSSON states that rye is sometimes attacked by Cladosporium, and that the mould, consumed in rye-bread or in beer, may give rise to diseases in the human being.

Concerning these, or at least closely-related, forms, ZOPF described exact morphological investigations accompanied by numerous illustrations in his memoir on Fumago, and also in his work on the fungi. These black, dew-like fungi just mentioned occur very frequently on parts of plants. FRANK correctly says:—"We are still quite in the dark with regard to specific differences, the reason of which is especially to be found in the frequent *polymorphism* of these organisms, and in the fact that the different evolution-forms are scarcely ever found together."

Among the various fungi occurring on the vine the two following parasites have obtained an unenviable notoriety:—

Oïdium (*Erysiphe*) *Tuckeri*, or "genuine mildew," forms whitish spots on the leaves and shoots of the vine, which later assume a brownish tinge. These consist of mycelium filaments from which separate elliptical or oblong, colourless conidia, 8 μ long and 5 μ thick. The mycelium spreads over the fruit, which is gradually covered with a tender growth of a grey colour, while it thrusts through the fruit skin roundish suckers, causing the epidermis cells to die. When grapes are attacked in a younger state, the epidermis is unable to keep up with the growth of the interior; it then gradually splits

open like skin affected by scurf; and the grapes either dry up or putrefy. They are capable of imparting to wine a very unpleasant smell and taste.

On mature grapes the fungus does not do so much harm, but it is apt to check their growth. The best remedy for this

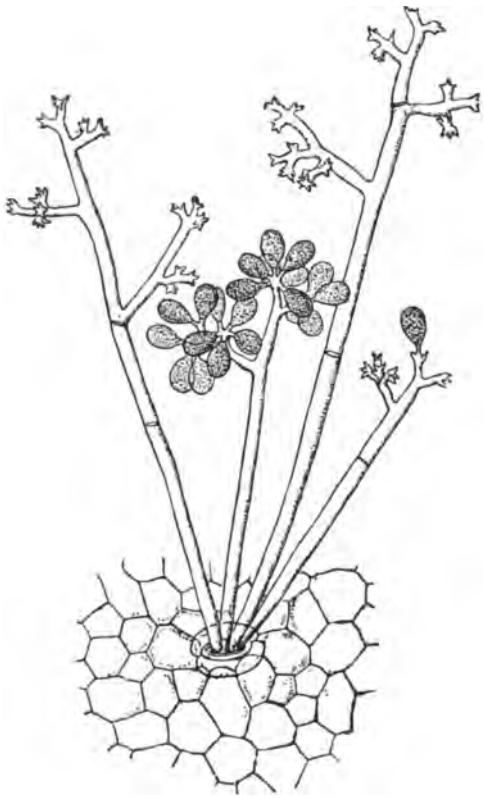

FIG. 36.—Peronospora viticola (after CORNU).

dangerous parasite is sprinkling with finely powdered sulphur, but this only takes effect in sunny weather.

The second vine fungus is "false mildew," *Peronospora viticola*, which penetrates into the interior of leaves and fruit, where it spreads and kills the cells. The conidia-carriers (Fig. 36) burst out from the stomata of the leaves in tufts.

On the upper part of the stem ramifications appear, both the branches and the principal axis ending in short conical apices. The conidia are egg-shaped, 12-30 μ long, and are provided with a smooth, colourless membrane. In the conidia, as a rule, 5-6 swarming spores are formed, which, when the conidia are immersed in water, burst out, penetrating through the epidermis of the leaves and grapes. The growth forms thick, prominent, whitish spots on the leaves and fruit. In the interior of the plant big, globular oospores (30 μ diameter) are formed, which have a brownish membrane, smooth or somewhat fluted, and are surrounded by the thin, colourless or yellowish oogonium wall. This fungus causes great injury, in that the grapes, according to the stage at which they are attacked, either wither away or putrefy; moreover, it destroys the foliage. This species is indigenous to North America, and was introduced into Europe in the year 1878 along with American vines; it has now spread to all vine-growing countries. Vine growers are endeavouring to suppress this pernicious parasite by the application of copper sulphate and similar remedies.

CHAPTER V.

ALCOHOLIC FERMENTS.

INTRODUCTION.

IT does not lie within the scope of a work of this description to give a detailed summary of the knowledge of bygone times; it will suffice to pass in review as much only as is necessary for the proper understanding of the present position of the subject under discussion. As the investigations of the last decade originated essentially from questions connected more or less directly with practice, the results obtained are also fully entitled to a practical application. It is evident, however, that this can only be brought about when the essential results of these scientific investigations are thoroughly appreciated; and it is with the object of facilitating this that the following *résumé* is given.

The term *alcoholic ferment*, as commonly used, is very comprehensive. Mould-fungi, as well as bacteria and budding-fungi, are able to induce alcoholic fermentation; but here we have only to deal with the last-mentioned. Among these *budding-fungi* are *some which also develop mycelium*, while *with others this form of growth does not as a rule occur; among the latter an important group is included under the name Saccharomycetes, on account of the property which its members possess of forming endogenous spores.*

In the year 1839 SCHWANN found that in the case of certain yeast-cells new cells were formed in their interior, and that these were liberated through bursting the walls of the mother-cells. J. DE SEYNES (1868) was, however, the first

who distinctly described the spores in yeast-cells. Shortly afterwards, in the year 1870, REESS proved that the formation of spores occurred in several species of yeast, and stated that the germination of these endogenous cells took place by budding. As far as the very imperfect methods of experimenting of that time permitted a conclusion being drawn, it appeared probable that there was a separate group of such budding-fungi, and to this group REESS gave the name *Saccharomyces*.[1] The conditions favourable to the formation of such reproductive organs in the cells were, however, unknown; there was no definite method by means of which their formation could be insured, and experiments having this for their object were made at random. In the work already quoted REESS also proposed a system for the classification of the *Saccharomycetes*, which he based solely upon the size and form of the cells. Such a classification founded upon purely microscopical appearances has, however, proved to be useless; it is impossible to distinguish between the different species by means of the characters indicated by REESS. His work has consequently been of no real practical importance; and since the essential conditions for the formation of spores were unknown to him and to his successor ENGEL—so that it was purely a matter of chance whether, in a culture of *Saccharomycetes*, spore-forming cells were obtained or not—it is easy to understand the doubt subsequently expressed as to the existence of spores, and the disputes which followed as to whether the yeast used in practice had or had not lost the property of forming spores. Finally, BREFELD believed he had definitely proved that cultivated yeast was completely deficient in this property. This confusion was at last dissipated and order established when HANSEN discovered the conditions regulating the formation of spores, and upon this basis for the first time devised *a method* for obtaining them.

PASTEUR'S "Études sur la bière" was published in the year

[1] The same author was, however, less consistent when he admitted into this group other kinds which did not yield spores, and in this he was also followed by DE BARY in "Comparative Morphology and Biology of the Fungi, Mycetozoa and Bacteria," Oxford, 1887. REESS thus, at once, destroyed the very system, the construction of which he had just undertaken.

1876, and this work advanced in many directions our knowledge of the phenomena connected with fermentation. The main portion of this book is devoted to the doctrine that every fermentation and every putrefaction is brought about by micro-organisms, a doctrine which he had defended with great force in earlier papers. PASTEUR'S name is with justice associated with this important doctrine, since it was mainly through his experiments that its truth has been confirmed and recognised. The idea, however, can be traced much further back. LINNÉ and others expressed the belief that the processes of fermentation and putrefaction were caused by living microscopic organisms; but proof was not forthcoming until much later. It has already been mentioned that in 1835 MITSCHERLICH and, later, CAGNIARD-LATOUR proved that the yeast of beer and wine consists of cells which reproduce themselves by budding, and that these cells bring about alcoholic fermentation. Shortly afterwards SCHWANN arrived at the same conclusion. In 1838 the view was expressed that different fermentations were caused by different micro-organisms; and it was about this time that TURPIN stated that there was "no decomposition of sugar, no fermentation without the physiological activity of vegetation." Reference may be made to the previous exposition of this doctrine, which in its historical development is so closely related to the doctrine of spontaneous generation (see "Sterilisation").

Important discoveries never originate from a single man, but really result from the work of many investigators; it is usually, however, much easier to conceive the idea of some truth than to furnish sufficient proof of its correctness. Thus, although the doctrine was not new, when in 1857 PASTEUR commenced his experiments, some very essential connecting links were wanting, as is evident from the fact that LIEBIG again gave preference to STAHL'S experiments in support of the chemical theory of fermentation. The victory gained by PASTEUR in this dispute constitutes the foundation of his great fame.

In his "Études sur la bière" PASTEUR clearly and incontestably proves the *significance of micro-organisms*, and he lays much stress upon the marked influence which bacteria are

capable of exercising upon fermentation and on the character of the resulting beer. He also treats of the budding fungi; and in the case of some imperfectly described members of this group, he intimates, as BAIL and others had done previously, that they affect the character of the products of fermentation in various ways. In this PASTEUR is merely repeating the indistinct views of previous investigators, and *his suggestions take two opposite directions.* This is distinctly seen in his observations on the so-called caseous yeast and the aërobic yeast. It is possible that in this case he may have been dealing with distinct kinds of yeast, but it is also possible that they were merely forms of ordinary brewers' yeast modified by some treatment to which they had been subjected. It must not, however, be overlooked that PASTEUR even pointed out the reason why the question could not be decided, namely, that *it was not then possible to determine whether he was dealing at the starting-point with one or with several species. An accurate method for the pure cultivation of the different kinds of yeast had not then been discovered* (compare Chapter I., "Preparation of the Pure Culture"). A true orientation in the world of micro-organisms is consequently not found in this work, and it is not possible in any part of PASTEUR'S statements to find such characteristics for the budding-fungi on which a scheme of analysis could be based. PASTEUR classed with the *Saccharomycetes* all the budding-fungi which showed any marked power of producing alcoholic fermentation, and it is nowhere clear whether his descriptions apply to *true Saccharomycetes* or to *other budding-fungi.* These yeast-fungi, which in our present system may belong to very different classes, were further in his book regarded as stages of development of mould-fungi resembling *Dematium*, but no evidence was given in proof of this view. Whether or not there are different species of these budding-fungi (*Saccharomycetes, Torula, Dematium*) PASTEUR leaves undetermined. His treatment of the botanical problems mentioned must be regarded as having essentially broken down.

The paramount reason why this work was not able to bring about the reform in brewing indicated in its preface was that from the position of science at that time it was impossible to

see clearly into the relations of the different alcoholic ferments occurring in fermenting liquids. PASTEUR was therefore unable to get beyond the indefinite conjectures and contradictory views of his predecessors. In his review of the micro-organisms which cause diseases in beer, he speaks only of *bacteria*; and the view that these are the only causes of diseases in beer has since been repeatedly expressed by DUCLAUX in 1883, and by other French, English, and German writers. PASTEUR, basing his views on these studies, recommended brewers to purify their yeast; and in order to free it from bacteria, advised its cultivation in a sugar solution containing tartaric acid, or in wort containing a little phenol (see below).

In contradistinction to this, HANSEN, in the year 1883, enunciated his doctrine that some of the *most dangerous and most common diseases of low-fermentation beer were caused, not by bacteria, but by certain species of Saccharomyces*, and that each of the names employed by REESS, namely, *Saccharomyces cerevisiæ, Sacch. Pastorianus, Sacch. ellipsoideus*, represented not one but several different kinds or races. He showed that varieties which until then had been incorrectly grouped under the one name *Saccharomyces cerevisiæ* gave products in the brewery having different characters. Starting from this, HANSEN elaborated his method, by means of which a pitching-yeast, consisting of *only one species*, is employed. After some resistance this system has been recognised and introduced into practice in all countries where the brewing industry is carried on. VELTEN of Marseilles, who formerly worked with PASTEUR, has, however, attacked this system (1887-89), the mistake of which he deems to be that HANSEN'S yeast consists only of one species. He considers it an advantage in PASTEUR'S purified yeast that the latter consists of several different kinds, and regards this combination of various species as necessary in order that the beer may acquire the desired taste and bouquet. HANSEN'S latest investigations show how completely this doctrine breaks down. HANSEN proved by experiment that when yeast is treated with tartaric acid, according to PASTEUR'S method, the *conditions are so favourable for the development of the yeasts producing disease, that finally the culture-yeast is completely suppressed.* PASTEUR subsequently greeted HANSEN'S

method as an advance, in that he wrote, "HANSEN was the first to perceive that beer-yeast should be pure, and not only as regards microbes and disease-ferments in the narrower sense, but that it should also be free from the cells of wild yeasts."

As, however, PASTEUR'S work always retains its technical importance, on account of the force with which the influence of bacteria in the fermentation industries is asserted, so it also possesses great theoretical interest, especially from the new theory of fermentation enunciated therein, which at the time necessarily attracted much attention.

Contrary to BREFELD, who asserted that yeast could not multiply without free oxygen, and TRAUBE (1858), who indeed granted that yeast was able to develop without free oxygen, but maintained that it then required for its cell-formation soluble albuminoids in the liquid, and that the activity of the yeast-cell as an alcoholic ferment depends on chemical combination, an enzyme being contained in the plasma, PASTEUR stated that the organisms of fermentation constitute a group of living beings, whose function as ferments is "a necessary consequence of life without air, of life without free oxygen"; and, further, that such a fermentation can take place in a pure sugar solution. He maintains that the reason why BREFELD could not get yeast to develop in a moist chamber in an atmosphere of carbon dioxide was because he was working with old yeast-cells, whilst it is only possible for yeast to multiply in the absence of free oxygen when the cells are very young. The minute quantity of free oxygen which is present in the liquid to which the yeast is added "rejuvenates the cells and makes it possible for them to resume the power to bud, to preserve life, and to carry on their multiplication without access of air."

Hence PASTEUR makes a distinction between two classes of organisms: *aërobic*, those which cannot live without the presence of free air; and *anaërobic*, those which can exist in the absence of air. According to his view, these latter constitute "ferments in the true sense of the word."

It would be incorrect to assume that the presence of alcohol and carbon dioxide among the products of the fermentation unconditionally presupposes the influence of "organisms of

alcoholic fermentation in the true sense of the term." The researches of LECHARTIER and BELLAMY, which were subsequently extended by PASTEUR, have shown that when grapes, oranges, and other fruits on which no yeast-cells were present, were preserved in vessels filled with carbon dioxide, a development of alcohol and carbon dioxide took place. "The fermentative character is consequently not a condition of the existence of yeast; the fermentative power is not peculiar to cells of a special nature, is no fixed structural characteristic, but is a property which is dependent upon external conditions and upon the mode of nutrition of the organism" ("Études sur la bière," page 258).

"In short, fermentation is a very general phenomenon. It is *life without air*, *life without free oxygen*; or more generally still, it is the necessary result of chemical work carried out on a fermentable substance, which by its decomposition is capable of evolving heat; the heat necessary to effect this work being borrowed from a part of that which is liberated by the decomposition of the fermentable substance. The class of fermentations properly so-called is limited by the small number of substances which are capable of evolving heat on decomposition, and which will serve as nourishment for the lower organisms when the admission of air is excluded" ("Études sur la bière," page 261). This is, briefly, PASTEUR'S famous theory of fermentation.

Fermentations dependent upon oxidation—such as the acetic acid fermentation, which, as PASTEUR himself had observed, requires an abundant supply of air—were consequently not regarded by him as true fermentations. It is seen, moreover, that he does not strictly adhere to his definition, in that he emphasises the fact that yeast also possesses fermentative properties when air is present, although to a less degree than when oxygen is excluded. The correctness of this under certain conditions has been confirmed in the case of bottom yeast by PEDERSEN (1878), and by HANSEN (1879), who came to the conclusion that the amount of substance in a wort which a definite quantity of yeast can convert into alcohol and carbonic acid is smaller when the liquid is aërated during fermentation than when no aëration takes place. ED.

BUCHNER (1885) obtained a similar result in his experiments with bacteria.

HANSEN arranged his experiments in such a way that a rotatory motion was imparted to the liquid which was being aërated, the cells thus being brought into continual contact with the vigorous current of air which was blown through the fluid. Nevertheless, there was a distinct alcoholic fermentation, and it certainly follows that this was not induced by life without air.

In NAEGELI'S "Theorie der Garüng" (1879) it is shown that the admission of oxygen is highly favourable to alcoholic fermentation in a sugar solution when no other nourishment is present, and consequently the yeast does not multiply, or does so only to a small extent. NAEGELI therefore states (p. 26) that "PASTEUR'S theory, that fermentation is induced through want of oxygen, in that the yeast-cells are forced to take the necessary supply of oxygen from the fermentable substance, is refuted by all the facts which bear upon this question."

A. J. BROWN, who also holds this view, made a series of experiments in which fermentations were conducted in presence of an abundant supply of oxygen, whilst in a duplicate set of experiments conducted simultaneously, oxygen was excluded; the same number of non-multiplying yeast-cells were present in both cases, and all the other conditions were kept constant. These experiments showed—contrary to PASTEUR'S theory—that the yeast-cells exercised a greater fermentative power in the presence of oxygen than when the latter was excluded.

BROWN emphasises the fact that PASTEUR'S theory rests upon comparative fermentation experiments, from which he draws the inference that the *fermentative power of the ferment* (*le pouvoir du ferment*)—that is, the proportion of the weight of yeast formed to the weight of fermented sugar—is very high when air is excluded, and very low if air is admitted. Against these experiments BROWN raises well-founded objections. PASTEUR, in his determination of the fermentative power, leaves out of account the *duration* of the fermentation ("Études sur la bière," page 245), so that he contrasts fermentations which had lasted only a few hours with those lasting several months. PASTEUR states that yeast possesses great *activity* (*activité*) if it has oxygen at its disposal, and that in this case it is able

to decompose a large amount of sugar within a short time; thus, the activity or energy is expressed by the amount of sugar fermented in unit time, and determined as the proportion between the weight of decomposed sugar on one hand, and the weight of yeast and time elapsed on the other. If the fermented sugar is termed S, the amount of yeast, l, and the duration of the fermentation, t, then, according to PASTEUR, the fermentative power $= \frac{S}{l}$, and the activity or energy $= \frac{S}{lt}$. But the activity of the yeast-cell is continuous, and the quantity expressed by $\frac{S}{l}$, therefore represents the amount of sugar fermented for the total number of time units during which the fermentation lasted. Therefore the duration must necessarily be taken into account in comparisons made for the purpose of determining the fermentative power of yeast (*pouvoir du ferment*); for the duration decides the results of the experiment according to which PASTEUR determines the fermentative power, and upon which he builds up his theory; but as he does not reckon with the time elapsed, it follows that his determinations must be incorrect, annihilating the foundation of the theory. Thus, PASTEUR'S "power of the ferment" and "activity" of the yeast in reality amount to one and the same thing.

In PASTEUR'S experiments, however, the real determinations of time which were requisite for establishing the total power of the ferment could not be made, because the amount of sugar upon which the yeast had to react was too small. When BROWN carried out a fermentation according to PASTEUR'S experiments, with a supply of air, until the original amount of sugar had been fermented, and when he then added fresh amounts of sugar, the yeast was able to ferment three times as much sugar as there was originally present, without any increase of weight of the yeast taking place. It also deserves notice that PASTEUR used cane-sugar, which had previously to be inverted; this inversion, which does not depend on the fermentative function, could only take place slowly under the conditions of PASTEUR'S experiments, and he ought to have allowed for the time taken in inversion.

BROWN also points out the fact that none of PASTEUR'S

results is incongruous with the opposite view of the nature of fermentation, namely, that the yeast-cell exercises its functions independently of its surroundings, *e.g.* of the presence or absence of free oxygen.

HUEPPE and his pupils have also opposed PASTEUR'S theory, and have brought forward examples of fermentation organisms " which can induce the specific fermentations even more readily when atmospheric oxygen is present."

Of NAEGELI'S manifold work on the lower organisms, we will only mention, as connected with the foregoing, the " molecular-physical " theory of fermentation put forward by him, which is essentially a modification of LIEBIG'S theory. Whilst PASTEUR explains fermentation as the result of activity occurring within the cell, NAEGELI defines fermentation as a transference of the vibrations of the molecules, groups of atoms and atoms of different compounds (which themselves suffer no change), contained in the living plasma to the fermentable substance, whereby the equilibrium of its molecules becomes disturbed and their decomposition brought about. In the process of fermentation, the vibrations of the plasma molecules are thus transferred to the fermentable substance. The active cause of fermentation is present in the living plasma, and therefore in the interior of the cells; but it operates at a moderate distance outside the cell. The *decomposition of sugar* into alcohol and carbonic acid takes place to a small extent within, but *mainly outside the yeast-cells.* This theory is thus distinctly opposed to that of PASTEUR, and follows on the lines of the theories propounded by STAHL and LIEBIG.

IWANOWSKY, on the basis of experiments made with absolutely pure cultures, holds the opinion that the supply of oxygen during fermentation exerts no influence on the fermentative power of yeast; he found that the same quantity of yeast fermented the same amount of sugar, whether the fermenting liquor was agitated by means of a strong blast of air or whether the fermentation took place in pure nitrogen; according to this observer the alcoholic fermentation performed by the yeast-cell depends simply on the *composition* of the *nutritive liquid.* He looks upon this function as the result of a diseased state which has been brought about during

the nutrition of the yeast-fungus, due to the abnormal composition of the liquid. The normal nutritive liquid contains a small amount of sugar and about twice as much peptone; in such a liquid the yeast multiplies freely, and feeds like other fungi without exciting any *true alcoholic fermentation.* On the other hand, he asserts that the so-called "*molecular respiration*"—in which, among other substances, alcohol is produced —depends in yeast, as in other plants, on the absence of free oxygen, and that this function manifests itself plainly if yeast is developed in a liquid containing but a small amount of sugar.

GILTAY and ABERSON, on the contrary, have confirmed the results of PEDERSEN and HANSEN, showing that a given weight of yeast produces more alcohol in non-aërated cultures than in aërated. They presume that the fact of yeast multiplying more freely when air is blown in may depend on the *movement* set up in the culture liquid, and on the consequent improved nutrition of the yeast-cells.

EMIL FISCHER has, by purely chemical research, resulting in his celebrated work on the *synthesis of the sugars,* on the use of phenyl-hydrazin, and the osazone-reaction, diverted the current views on fermentation phenomena into new channels. His researches led him to explain the behaviour of the yeast-cell towards the particular sugar of the nutritive liquid in the same way as the action of the enzymes (invertase, emulsin), so that the chemical activity of the living cell does not differ from the action of chemical ferments. According to FISCHER, *fermentation of polysaccharides is always preceded by hydrolysis of the sugar.* But there exists an exact relation between the molecular structure of a given sugar and the sugar inverting enzyme of a yeast-cell; if a sugar comes into contact with the albuminoids of a yeast-cell, which play the most important part among the agents of which the living cell makes use, the sugar is decomposed only if its *configuration,* the *geometrical structure* of its *molecules, does not deviate too much from the configuration of the molecules of the albuminoid.* Thus, according to FISCHER'S theory, the function of the living cell depends much more upon the molecular geometry than on the composition of the nutritive material.

Another way in which FISCHER, and also THIERFELDER,

obtained confirmation of this fermentation theory was by examining the behaviour of HANSEN'S and other yeast species towards the artificial sugar species, synthetically obtained by FISCHER. They found, indeed, that the yeast species are quite fastidious regarding the geometrical configuration of the sugar molecule, whilst they often remain unaffected by other alterations in its composition.

Among the various synthetically prepared sugar species examined by FISCHER with regard to their behaviour towards yeasts, *melibiose* is especially mentioned. It is fermented by brewers' common bottom-fermentation yeasts, but not by many brewers' top-fermentation yeasts. In harmony with this, FISCHER found that bottom-fermentation yeast contains an enzyme capable of extraction from the dried yeast in aqueous solution, which decomposes melibiose, converting it into glucose and galactose; but in a corresponding treatment of the brewers' top-fermentation yeasts used no decomposition of this sugar could be observed. As brewers' top-fermentation yeast contains invertase, it follows that the ferment which splits up melibiose cannot be identical with invertase.

C. J. LINTNER and FISCHER showed, by methods devised by the latter, that natural *maltose*, if acted upon by an aqueous extract of dried yeast, is split up into glycose, and that there is a marked difference between this enzyme and invertase which decomposes cane-sugar. FISCHER terms the former enzyme yeast-glycase or yeast-maltase. Its optimum temperature is about 40° C., whilst that of invertase, according to KJELDAHL, is 52°-53° C.

PRIOR compared the *fermentation energy* of a number of yeast-cells by accurately testing them under similar conditions according to MEISSL'S method. He found marked differences, as shown in the following examples. The numbers are calculated on the weight of dried yeast.

Carlsberg yeast,	I.,	- -	136·40
,, ,,	II.,	- -	106·13
Saccharomyces Pastorianus,	I.,	- -	153·48
,, ,,	II.,	- -	280·72
,, ,,	III.,	- -	202·20
,, ellipsoideus,	I.,	- -	285·76
,, ,,	II.,	- -	219·03

PRIOR is of opinion that the different behaviour of yeast species in this respect may be partly accounted for by the supposition that the *cell-walls* are not equally thick in different species, and that the *permeability varies*; he assumes that the thickness of the outer slime layer of the cell-wall also plays a part. From comparative experiments with the same type of yeast, which he made to act upon different sugar species, he further concludes that *the individual sugar species possess different rates of diffusion through the cell-wall*, the diffusive power of cane-sugar being greatest, that of maltose least.

We may aptly conclude this survey with a brief mention of the preliminary communications of ED. BUCHNER on the *separation of the active ferment from the living yeast-cell.* Common brewers' yeast, mixed with quartz sand and kieselguhr, was finely ground, mixed with water, and then subjected to a pressure of 4-500 atmospheres. The expressed juice, containing substances extracted from the contents of the yeast-cells, is capable of quickly fermenting highly concentrated sugar solutions of various kinds, even if the juice is filtered through kieselguhr (as in a Berkefeld filter), and the mixture of expressed juice and sugar solution saturated with chloroform. BUCHNER infers from his experiments that the fermentative power of the expressed juice is embodied in a soluble enzyme-like substance isolated from the living cell-plasma, undoubtedly an albuminoid, which he terms *zymase.* If allowed to stand, this expressed juice soon loses its power, whereas if mixed with a 75 per cent. saccharose solution, it retains its activity for a long time. On evaporating and drying the expressed juice, a brittle, yellowish mass is obtained, which is capable of exhibiting fermentative activity for a considerable time.

Thus, BUCHNER'S results harmonise with the chemical theories of TRAUBE and FISCHER, alluded to above.

RAYMAN and KRUIS have added to our knowledge of the biology of yeast-fungi by their experiments on beers which, for several years, had undergone fermentation with absolutely pure cultures, prepared by HANSEN'S method. These investigators found that the fermentation product obtained by means of pure cultures of *Saccharomycetes*—all the normal conditions of temperature, etc., obtaining in the brewery being maintained

—is a single alcohol, namely, *ethyl-alcohol.* This alcohol, together with the living yeast, will exist unaltered for years in the beer when the latter is preserved at a low temperature and air is excluded; when, on the other hand, a yeast film is allowed to form on the surface through the admission of air, a vigorous oxidation sets in, and the *alcohol is converted into carbon dioxide and water.* According to later researches made by the same authors, the culture-yeasts in distilleries, under certain conditions, probably when the cells are in a state of exhaustion, are capable of forming amyl-alcohol, acetaldehyde, and furfurol; the acetaldehyde is supposed to be formed through oxidation of ethyl-alcohol in the nascent state. In prolonged fermentations the *Saccharomycetes* hydrolyse the albuminoids present in the nutrient fluid to a variable extent; they can also oxidise the products to formic and valerianic acids. The same authors distinguish two reactions in normal fermentations, namely, a sugar-hydrolysing reaction taking place in the nutrient medium, and a synthetic (albuminoid) reaction taking place in the interior of the organism. They regard fermentation as an alternate hydration and dehydration.

GENERAL REMARKS ON HANSEN'S INVESTIGATIONS.

From the above *résumé* it will be seen that, at the time when HANSEN commenced his investigations, our knowledge of the alcoholic ferments was very deficient and untrustworthy. From the foundations upwards, it was necessary to attack the whole problem experimentally. For years past, HANSEN has steadily engaged in this work.

Previous investigators had certainly travelled as far as was possible along the paths which they had marked out. When we compare their investigations—especially those of PASTEUR and REESS—with those of HANSEN, we find that the latter attacked the problem from new points of view and with new methods. He extended his investigations on this subject far and wide. His researches have not only opened up new fields for scientific research, but they have also brought about a reform in the fermentation industry. For these reasons it is

only right that they should form the ground-work of the following section of this book.

When HANSEN published, in 1878, his treatise on "Micro-Organisms in Beer and Wort," he pointed out the uncertainty which prevailed in the works of earlier writers, concerning the true *Saccharomycetes*; and asserted that it was impossible to proceed further along the path which they had pursued, but that the investigations (especially those commenced by PASTEUR and REESS) must *be attacked from a totally different point of view* if they were to lead to any definite issue. It was only in the latter end of the year 1881 that he succeeded in finding the key to the solution of the problem. In the first place the problem was to devise means by which growths could be obtained, each of which was derived from *a single cell*, in order to determine by experiment *whether these incontestably pure cultures exhibited constant characters*—that is to say, how far the *Saccharomycetes* occur as species, varieties, or races—and, should this prove to be the case, to determine what these characters are. When this problem was solved, the next was to devise a method for the analysis of yeast and to study the conditions of life of these organisms.

1. *PREPARATION OF THE PURE CULTURE.*

Reference was made in our first chapter to the prevalence of the idea that the one condition for an exact knowledge of micro-organisms, hundreds or thousands of which we find in every drop when examined under the microscope, consists in the isolation of a single cell, and subsequent study of a pure growth obtained from this cell. The different methods which had been employed were also briefly described.

HANSEN has repeatedly pointed out that *the only inevitably sure method is to start from the individual cell and to secure the beginning from this.* He has devised two different methods for this purpose. In his first method a liquid medium was employed, and in his second method a solid medium, for the cultivation; in both cases the culture had been diluted as previously described (Chapter I., "Preparation of the Pure Culture").

With the help of the acquired knowledge of the species it was possible to submit these methods to a searching examination, with the result that they proved to be reliable.

If it is desired to isolate from a mixed growth of different species those which are in an *enfeebled condition*, it is necessary, as HANSEN points out, to employ the dilution method, using a suitable nutrient fluid, such as wort, the conditions being then favourable for the growth of the organisms in question. The yeast is introduced into flasks containing the liquid, or into drops, each of which is kept in a moist chamber.

If, on the other hand, we wish to separate from a mixed growth a species which is *in a vigorous state of development*, and whose further growth is consequently not dependent upon specially favourable conditions of nutriment, we can attain our object more readily and in a shorter time by the employment of a solid nutrient medium—in this case gelatine and wort.

It has been proved that the addition of gelatine to wort diminishes its value as a nutritive material for the yeast-fungi. A series of experiments carried out by HOLM show in fact that, if some of these cells are introduced into wort-gelatine at the commencement of a fermentation, when the yeast-cells are in their most vigorous state of development, about 4 per cent. of those sown do not develop; if, on the other hand, the yeast-cells are taken at the conclusion of a fermentation, when they are enfeebled, about 25 per cent. give no colonies in wort-gelatine.

The advantage of this method, as employed by HANSEN, for the study of budding-fungi is that it makes it possible to *directly observe individual cells under the microscope* and to *follow their further development*, for the gelatine plate or the drop is enclosed in a moist chamber (compare Chapter I., "Dilution Methods").

2. *THE ANALYSIS.*

Throughout the entire series of HANSEN'S researches a leading idea obtains, namely, that *the shape, relative size, and appearance of the cells, taken by themselves, are not sufficient to characterise a species*, since the same species, when exposed to

different external conditions, can occur in very varied forms and may differ greatly in appearance. On the other hand, the forms of development of the cells, regarded from another point of view, constitute very important distinctive characters for different species. Thus it is found that *different species under the same treatment behave differently and assume different forms.* This can only be explained by assuming that there are intrinsic, natural characters in the special cells which exert an influence of their own.

There follows a brief account of the various means by which HANSEN determined the characteristics of different species. These investigations form at the same time contributions to the general physiology of the budding-fungi.

(*a*) **The Microscopic Appearance of the Sedimentary Yeast.**—The first examination of a yeast will generally consist in observing the appearance of the *sediment* under the microscope. As examples illustrating what information may be gained in this way, we call attention to the figures 47, 50, 52, 54, 56, representing the young sedimentary forms of the six species of *Saccharomycetes* which have been specially investigated by HANSEN. The growths were obtained by cultivating the cells for some time in wort, then introducing fresh wort, and maintaining a temperature of 25° to 27° C. for 24 hours; thus a vigorous growth was developed. Comparing, for instance, the figures representing *Saccharomyces cerevisiæ I.* with those which illustrate the three *Pastorianus* species, we find that, *taken as a whole, they show marked differences. Saccharomyces cerevisiæ* consists mainly of large round or oval cells, the *Pastorianus* species form mostly elongated sausage-shaped cells. It is, however, a very different matter when cells of the first species are mixed with cells of one of the other species; it is then impossible, judging from the form alone, to distinguish the larger and smaller oval and round cells of the *Pastorianus* species from many of the cells *of Sacch. cerevisiæ.* The two species *Sacch. ellipsoideus I.* and *II.* consist mainly of oval and round cells; sausage-shaped cells, however, also occur; and thus it is in this case impossible to determine the species by the form of the cells when these are mixed with *Sacch. cerevisiæ* or *Sacch. Pastorianus.*

Neither can any conclusions be arrived at by direct measurements of these sedimentary forms.

A glance at these six figures of pure cultures shows that we have here *three different classes of budding-fungi*, one of which is represented by *Sacch. cerevisiæ*, whilst the second includes the three *Pastorianus species*, and the third the two *ellipsoid species*. This much, but only this much, is possible from a purely microscopical examination, and this only under the conditions of cultivation indicated.

(*b*) **Formation of Ascospores.**—By HANSEN'S work on the formation of endogenous spores in the *Saccharomycetes* the first essential of an *analytical method* for the examination of yeast was determined. We add a brief account of the experimental method adopted and of the general results obtained.

The formation of spores in yeast-cells had been investigated by various naturalists, but the only established result of their numerous and, to some extent, contradictory statements was the fact that *Saccharomyces cells could, under certain unknown conditions, form spores in their interior.*

After making a large number of experiments, HANSEN was able to determine the following *conditions regulating the formation of spores in the Saccharomycetes*:—

1. *The cells must be placed on a moist surface and have a plentiful supply of air.*
2. *Only young, vigorous cells can exercise this function.*
3. *The most favourable temperature for most of the species yet examined is about 25° C. This temperature favours spore-formation in all known species.*

A few *Saccharomycetes* likewise form spores when they are present in fermenting nutrient fluids.

A growth of yeast is developed in the way described on page 144. Older cultures, developed in saccharose-solution or in wort, must be cultivated several times in aërated wort before showing a normal formation of spores. A small quantity is transferred to a previously sterilised gypsum block; this block takes the shape of a truncated cone; it is enclosed in a flat glass dish covered by a larger inverted dish, and is

kept moist by half filling the dish with water.[1] If it is desired merely to bring about the formation of spores, the apparatus may be allowed to remain at the ordinary room-temperature.

HANSEN was the first to give an accurate description of *the structure of spores* and a detailed account of their *evolution founded upon observations of individual spores*; and he distinguished *three typically different groups of Saccharomycetes* which are characterised either by the mode of germination or by the form of the spores.

After a certain lapse of time, which varies with the different, species, roundish plasma-particles appear in the cells and these

FIG. 37.—The first stages of development of the spores of Saach. cerevisiæ I. (after HANSEN): *a*, *b*, *c*, *d*, *e*, rudiments of spores, where the walls are not yet distinct; *f*, *g*, *h*, *i*, *j*, completely-developed spores with distinct walls.

are the *first indications of spores* (Fig. 37). In their further development, they are surrounded by a *wall*, which is seen more or less distinctly in the different species.

In the *first type*, to which *Saccharomyces cerevisiæ I.* belongs, the spores may expand during the first stages of germination to such an extent that the pressure which they exert on each other while they are still enclosed in the mother cell, brings about the formation of the so-called partition walls (Fig. 38). This causes more or less plasma to be squeezed or wedged between the spores, or the walls of the spores may be brought into contact. During further development, a complete union

[1] *Ascospores* can also be obtained when yeast is spread upon sterilised solidified gelatine, prepared with or without a nutrient solution, kept in a damp place; likewise in yeast-water and in sterilised water; finally, spore-forming cells occur in the films of the *Saccharomycetes*. The method is evidently not dependent upon these different substrata but upon the knowledge of the factors which render it possible for the cells to exercise this function of forming spores.

of the walls may take place, so that a true *partition wall* results; the cell then becomes a compound spore divided into several chambers.

FIG. 88.—Spores of Sacch. cerevisiæ I. in the first stages of germination (after HANSEN): at *a*, *d*, *e*, and *g*, formation of partition walls; at *e*, *f*, and *g*, the walls of the mother cells have become ruptured; at *g* a compound spore divided into several chambers, the coherent wall is ruptured in three places.

During *germination* (Fig 39) the spores swell and the wall of the mother-cell, which, originally, was moderately thick and

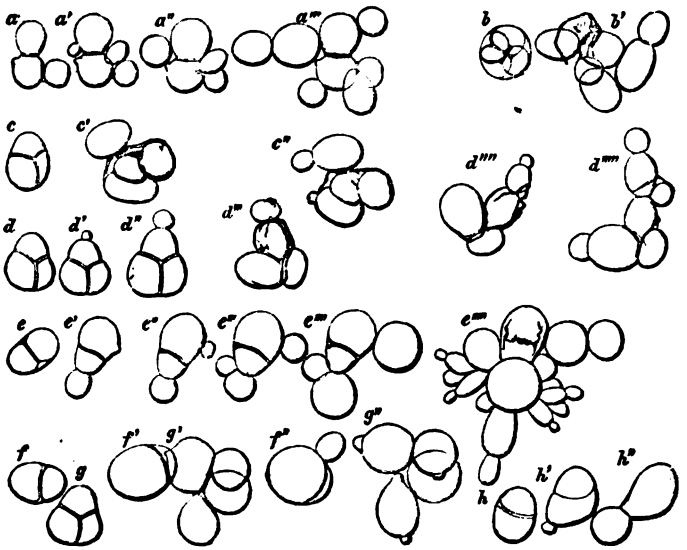

FIG. 89.—Budding of the spores in Saccharomyces cerevisiæ (after HANSEN): *a*, three spores without the wall of mother-cell; *b*, cell with four spores; at *b'* the wall of mother-cell is ruptured; *c*, cell with four spores, three of which are visible; at *c'* and *c''* the ruptured wall of mother-cell is seen; *d*, cell with three spores, at *d'''* the ruptured wall of mother-cell; *e*—*e''''* development of a very strong colony; *f*—*h*, other forms of development; at *h''* the wall between the two spores has disappeared.

elastic, stretches out and consequently grows thinner. It is finally ruptured, and then remains as a loose or shrivelled

skin, partially covering the spores; or it may gradually dissolve during germination.

Budding can occur at any point on the surface of the swollen spores; this budding usually takes place after the wall of the mother-cell has been ruptured or dissolved, but it also occasionally takes place within the mother-cell. After the

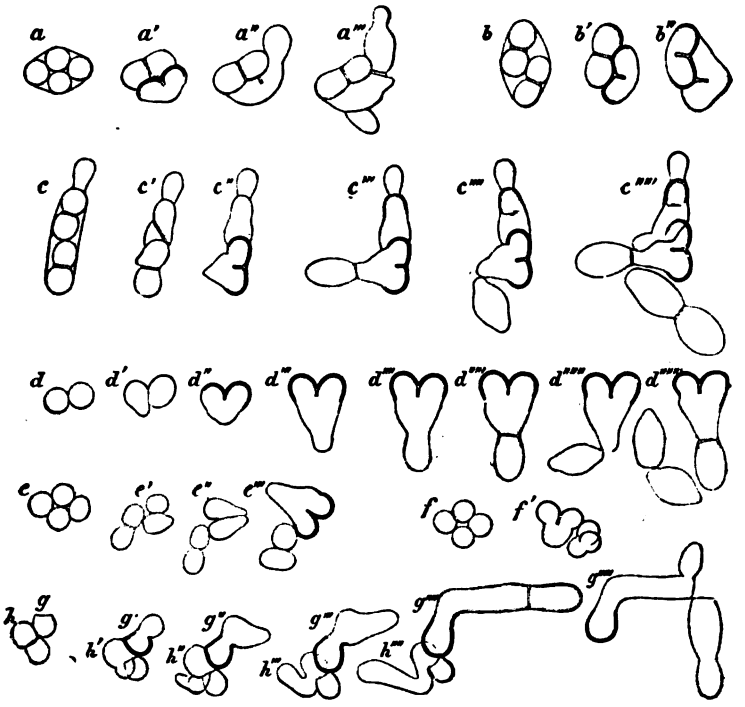

FIG. 40.—Germination of the spores of Saccharomyces Ludwigii (after HANSEN): *a*—*c* represent a gypsum-block culture 12 days old; *d*—*h*, a similar culture, one and-a-half months old.

buds have formed, the spores may remain connected, or they may soon be detached from each other.

An especially curious and exceptional case is that of the wall separating two spores being dissolved, so that a *fusion of the spores* results (see Fig. 39, *e*—*e*'''' and *h*—*h*''). HANSEN assumes that the biological significance of this phenomenon is that the spores, placed under unfavourable conditions, have a greater chance of forming buds. One spore plays the part of a

parasite to the other. The amalgamation of the spores is, perhaps, the beginning of the process.

The germination of the spores of those species of the groups *Saccharomyces Pastorianus* and *Sacch. ellipsoideus* which have been examined, takes place in essentially the same way as that just described.

A *second* and quite different type occurs in the case of *Sacch. Ludwigii* (Fig. 40), where *the fusion takes place in the very first stages of germination*; in this case, however, it is the *new formations* and not the spores which grow together. These new formations are further distinguished from the previous type in that they are not yeast-cells, but mycelium-like growths,—*promycelium.* The development of yeast-cells takes place from this *promycelium, a sharp partition wall being first formed*; the cell is then detached, and its ends are finally rounded. At the ends of these cells buds are developed, and these also split off at the partition walls.

In the case of the older spores this curious fusion is more

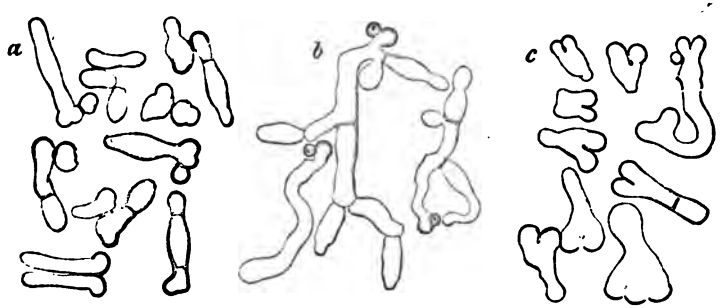

FIG. 41.—Saccharomyces Ludwigii (after HANSEN): germinating spores from old gypsum-block cultures; at *a* and *b* each spore has developed a germ-filament; at *c* are shown different forms produced by fusion.

uncommon (Fig. 41). Some germ-filaments develop into a branched mycelium (group *b*).

The *third type* which occurs in *Saccharomyces anomalus* (Fig. 42, see also description of the species), is distinguished from the former in that the spores are of *quite a different shape,* resembling the spores of *Endomyces decipiens.*[1] They are almost semi-spherical with a rim round the base.

[1]A fungus which is parasitic on the lamellæ of certain mushrooms.

During germination the spore swells and the projecting rim may either remain or disappear. Buds then make their appearance at different points on the surface of the spore.

One of the objects of HANSEN'S investigations was also to determine in what way the formation of spores was

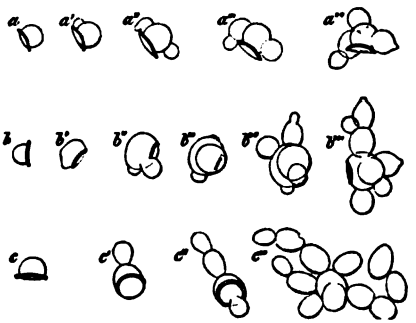

FIG. 42.—Germination of spores of Saccharomyces anomalus (after HANSEN).

influenced by different *temperatures*, with a view of ascertaining whether the different species behave alike, or whether it might not be possible in this way to discover different characteristics. It was, therefore, necessary to determine: First, *the limits of temperature, i.e.*, the highest and lowest temperatures at which spores could be formed; secondly, *the most favourable temperature, i.e.*, the temperature at which spores appeared in the shortest time; and, thirdly, the relation between *the intermediate temperatures.*

In determining the required intervals of time, the moment was registered at which *the cells showed distinct indications of the formation of spores* (compare Figs. 37 and 43). It is not possible to make use of ripe spores in these determinations, since no criterion exists for complete ripeness.

The results obtained by HANSEN are as follows:—

The formation of spores takes place slowly at low temperatures, more rapidly as the temperature is raised to a certain point; when this point is passed, their development is again retarded, until finally a temperature is reached at which it ceases altogether.

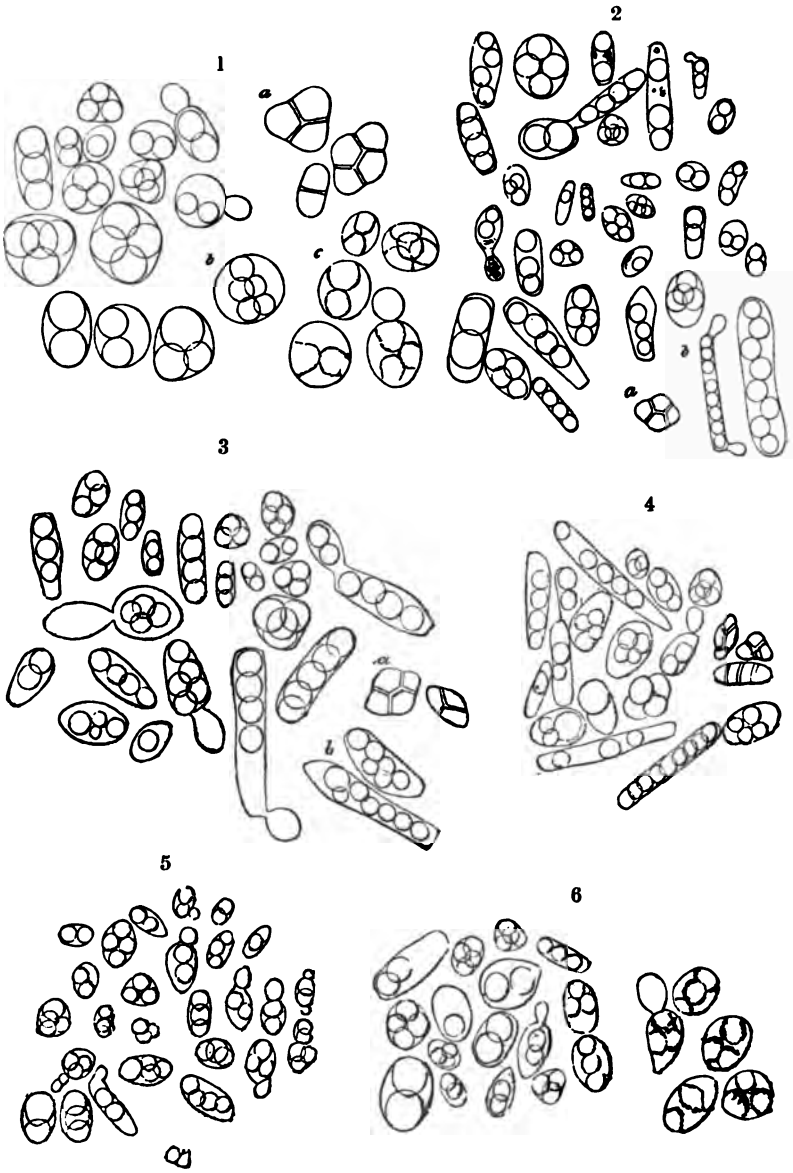

FIG. 43.—Saccharomycetes with ascospores (after HANSEN): 1, Sacch. cerevisiæ I.; 2, Sacch. Pastorianus I.; 3, Sacch. Past. II.; 4, Sacch. Past. III.; 5, Sacch. ellipsoideus I.; 6, Sacch. ellips. II.; *a*, cells with partition-wall formation; *b*, cells containing a larger number of spores than usual; *c*, cells showing distinct rudiments of spores.

The lowest limit of temperature for the six species first investigated was found to be 0·5° to 3° C., and the highest limit 37·5° C. HANSEN also determined the intermediate temperature and time relations for these six species, and found that when these two values are graphically represented, with the degrees of temperature as abscissæ and the time intervals as ordinates, the curves obtained for all six species had essentially the same form. They sink from the ordinates of the lowest temperatures towards the axes of the abscissæ, and then rise from these; at the same time, however, it is seen from these curves that the *cardinal points determined, more especially from the highest and lowest temperatures, give characteristic distinctions for the different species*; that is to say, *that the limits of temperature within which the formation of spores can take place are different for the various species* (compare classification of the Saccharomycetes).

With regard to the time required for the appearance of the first indications of spore-formation in *the six species* investigated, when maintained at the same temperature, the following was observed: At the highest temperature the time required for the development was for every species about 30 hours; at 25° there was also no great difference in the time required; *at the lower temperatures, however, marked differences occurred.* Thus, in the case of *Sacch. cerevisiæ I.*, the first indications of spore-formation at 11·5° C. are only found after ten days; but in the case of *Sacch. Pastorianus II.* after 77 hours.

In all determinations of this kind *a very considerable influence is exerted by the conditions of the cells*, the results vary according to the temperature at which they have grown, their age and vigour (compare the section in Chapter V. on the Variation of Yeast-cells). It follows from this that the composition of the nutrient fluid also exercises an influence. In methodical, comparative experiments of this nature, it is necessary, therefore, that the previous cultivation of the cells should always be carried out in the same manner. If these external conditions are varied, the limits for the reactions of the species corresponding to such varied conditions must likewise be determined.

By means of these experiments HANSEN has established an

important character for the determination of the *Saccharomycetes.*

A new distinctive characteristic for the species has been discovered by the same author in the *different anatomical structures of the spores.* Both these characters and others, which are described in the following pages (*e.g.*, film-formation), must necessarily be considered in a complete examination of a *Saccharomyces* species.

The method given below for the practical *analysis of low brewery-yeast* was based by HANSEN on certain observations both of the temperature curves for the development of spores and of the structure of spores. Thus it was found that at certain temperatures the species employed in the brewery, the so-called *cultivated yeasts, develop their spores later than the so-called wild yeasts,* several species of which also occur as *disease germs* in the brewery. HANSEN also found that *the structure of the spores in these two groups is generally different.* The young spore of cultivated yeast has a distinct wall or membrane, the contents are not homogeneous, but are granular, and exhibit vacuoles. In the case of wild yeast, on the other hand, the wall of the young spore is most frequently indistinct, the contents are homogeneous and strongly refract light. It should also be added that the spores of cultivated yeasts are usually larger than those of wild yeasts.

1. For the *continual, daily control of low brewery-yeast,* as regards contamination with wild yeast-species, the following very convenient method is made use of:—*At the conclusion of the primary fermentation,* a small quantity of the liquid is transferred from the fermenting-vessel to a sterilised flask; this is set aside for some hours until the yeast has settled to the bottom, and the sediment is spread upon a gypsum block in the manner described above. This is then introduced into a thermostat maintained at a temperature of 25° C. or 15° C.

It was shown that the species of cultivated yeasts employed in low-fermentation breweries can be divided into two groups. This has been subsequently confirmed by the elaborate investigations of HOLM and POULSEN. *One group* yields spores at a later period than wild yeast, when a temperature of 25° C. is maintained; *the other group,* on the contrary, produces spores in

about the same time as wild yeast at the above temperature, but at a temperature of 15° C. the cells of wild yeast show spore-formation considerably sooner than the cells of these cultivated yeasts.

The cultures maintained at 25° C. are examined after an interval of 40 hours, and those maintained at 15° C. after an interval of three days.

Experiments made by the author show that *high brewery-yeasts* can be analysed in a similar manner. In the case of some species, however, the analysis is best made at 10-12° C., because a well-marked difference of time between the beginning of spore-formation in culture-yeast on the one hand and wild yeast on the other is only observable at this temperature.

According to the author's researches, *distillers' yeast* may be analysed in the same manner. Rather low temperatures are usually preferable for this analysis. Often, however, the investigation into the *construction of the spore* in the selected yeast-type must form the chief part of the analysis, the difference of time for spore-formation in culture-yeast and wild yeast respectively frequently proving inadequate.

Wine yeast, like beer yeast, may be analysed by HANSEN'S method, as ADERHOLD has, more especially, emphasised.

By means of experiments undertaken to determine to what extent HANSEN'S analytical method can be relied on for technical purposes in low-fermentation breweries, HOLM and POULSEN concluded that *a very small admixture of wild yeast, about* 1-200*th of the entire mass* (Carlsberg bottom-yeast No. 1), *can be detected with certainty in this manner.* HANSEN'S previous researches had shown that when, for instance, the two species, *Sacch. Pastorianus III.* and *Sacch. ellipsoideus II.*,—which are capable of producing yeast-turbidity in beer—are present to the extent of only 1 part in 41 of the pitching-yeast, the disease is *not* developed, provided that the normal conditions of fermentation and storage have been maintained. Further, *Sacch. Pastorianus I.*, which imparts to beer a disagreeable odour and an unpleasant bitter taste, can, under the same conditions, scarcely exert its injurious influence when the admixture of this yeast amounts to less than 1 part in 22 of the pitching-yeast. Consequently, HANSEN'S method for the

analysis of yeast by means of ascospore-formation gives ample information as to the presence of these disease ferments.

This method likewise possesses the advantage that *the analysis can be performed with mixtures similar to ordinary pitching-yeast, and that it can be performed in a short time.*

When the object of the analysis is to characterise with greater accuracy the different species present in the sample, a number of cells are isolated by *fractionation*, and each of the growths obtained is separately examined.

In an investigation of *bottom-yeast* in the different stages of the primary fermentation, published by HANSEN in 1883, it was shown that the young cells of wild yeasts are present in largest amount during the last stages of primary fermentation and in the upper layers of the liquid. *The samples taken from the fermenting vessel for the analysis of the yeast must, therefore, be taken during the last few days of the primary fermentation.* If considerable time has elapsed before an analysis is commenced, the yeast must be introduced into wort, and one or more *complete* fermentations carried out; and this applies to yeast supplied in either a dry or a liquid state.[1]

The rule that wild yeasts develop only in the more advanced stages of fermentation applies also to *top-fermentation yeast as used in breweries.* This was shown by numerous analyses of beer from Danish, English, French, and German breweries carried out in the author's laboratory. As is well

[1] This observation regarding low-fermentation yeast has been confirmed by J. VUYLSTEKE'S experiments, in which fermentations were carried out with mixtures of different *Saccharomycetes* in cylindrical glass vessels of about two litres capacity; by counting the cells and by means of cultures, the relative proportions of the different species were determined. VUYLSTEKE'S experiments have shown that when his conditions are adopted this rule is not generally applicable to the case of mixtures of high-fermentation yeasts with wild yeasts. In some experiments with mixtures of *Sacch. cerevisiæ I.* (HANSEN) and *Sacch. Pastorianus I.* (HANSEN), the wild yeast was found to have increased towards the end of the primary fermentation, whilst in other experiments a diminution of the wild yeast was observed. On the other hand, all the experiments with mixtures of *Sacch. cerevisiæ I.* and *Sacch. Past. III.* showed that the impurity was greater in the upper layers of the liquid at the end of the primary fermentation than at the commencement, just as in the case of bottom-fermentation.

known, it was this very appearance of wild yeast in English top-fermentation breweries which gave rise to the erroneous view that such species are necessary for conducting a normal secondary fermentation. The assertions advanced in other quarters that wild yeasts do not usually occur in any considerable quantity in top-fermentation breweries can only be attributed to incorrect observations.

It is evident, however, that, valuable as the analysis of yeast is, it must always remain of secondary importance in the brewery; the most important factor, whatever the conditions, will be the employment of a pure cultivation of a selected species of yeast.

2. The analysis of the yeast in the *propagating apparatus*, which *must be absolutely pure*, is carried out as follows:—At the conclusion of fermentation, samples are withdrawn, with every precaution, into Pasteur flasks or into the Hansen flasks employed for sending out yeast samples; from these, small quantities are introduced into flasks containing neutral or slightly alkaline yeast water or yeast-water dextrose, and these are maintained at a temperature of 25° C., the object being to test the yeast for bacteria. The remainder is set aside for the yeast to settle, the beer is decanted, and a sample portion of the sediment is introduced into a cane-sugar solution containing 1 to 4 per cent. of tartaric acid. After three or four cultivations in such a solution it is further cultivated a few times in beer-wort, and then tested for spore-formation. The smallest traces of wild yeast in the apparatus are brought into a state of vigorous development by this treatment (see Chapter I., Physiological Methods[1]).

(*c*) **The Formation of Films.**—By studying the formation of films, HANSEN has discovered an entirely fresh set of characteristics for the Saccharomycetes. Earlier information by various writers not being in accordance with facts, the credit is due to HANSEN of having opened up this field of research.

[1] It is evident that this method is not available for the analysis of ordinary yeast, because the cultivation in the tartaric solution will cause the wild yeast-cells to increase very considerably in number, and consequently render it impossible for the analyst to judge of the degree of contamination.

It is a widely-known phenomenon, that *fermented liquids become coated with films.* The films formed by the budding fungi—*Mycoderma cerevisiæ*, *Mycoderma vini*—have especially attracted attention. The frequent mention of such films in the literature of our subject led to a result well known in other branches of science; they had been referred to for so long as though well understood that at last the belief in the actual existence of this knowledge became firmly rooted. After HANSEN had submitted this question to an experimental investigation, he showed, however, that this view was erroneous.

HANSEN has dealt with a large number of films, and amongst them are several forms most closely related to different species of *Saccharomyces Mycoderma*, which do not produce endogenous spores. According to DE SEYNES, REESS, and CIENKOWSKI, these Mycoderma-species do yield ascospores; it is, however, highly probable that these investigators were dealing with impure films containing an admixture of true *Saccharomycetes.* It is, indeed, a matter of no little difficulty to determine the purity of such a culture if one does not start from a single cell; for if *Mycoderma cerevisiæ* is cultivated as sedimentary yeast, the cells assume an entirely different appearance from that of the film-cells; they are filled to a great extent with plasma, whilst the cells of the film are, as is known, poor in plasma and contain strongly-developed vacuoles. Such forms, which are generally regarded as *Mycoderma cerevisiæ*, readily and quickly form films; some simultaneously exhibit distinct signs of fermentation, whilst others do not. On beer and wort these films are grey and dry in appearance; afterwards they become wrinkled and lighter in colour; air is found freely intermixed between the cells. Some of the varieties of *Torula* investigated by HANSEN yield similar films; the film of *Chalara Mycoderma*, on the other hand, is glutinous, tough, and slightly lustrous. In the case of *Monilia*—which, as previously mentioned, may occur with budding cells—the film-formation is peculiar: even during vigorous fermentation a film forms on the bubbles of foam, spreads gradually over the whole surface, and sometimes becomes wrinkled. Thus, the cells in the flask first sink to the bottom as sedimentary yeast,

set up a vigorous fermentation, and again rise with the bubbles of carbon dioxide to the surface, where they enter upon a new phase of development. If sterilised lager beer is infected with this fungus, no fermentation sets in, and only a thin film resembling dust is developed; under other conditions the fungus forms a white, floury, or woolly layer, as in the case of *Oïdium*.

The true Saccharomycetes also form films, which, however, differ somewhat from those just mentioned; this is also the case with some of HANSEN'S *Torula and with Saccharomyces apiculatus.* From these observations it is evident that the formation of films is not a peculiarity of certain species, but must be regarded as *a phenomenon common to all micro-organisms.*

In the case of the *Saccharomycetes* this phenomenon generally occurs in the following manner:—If cultures in wort are left undisturbed for a longer or shorter time at the ordinary room temperature, small specks of yeast gradually appear on the surface of the liquid, after the termination of the primary fermentation; these can afterwards coalesce to figures of different forms and sizes—isolated patches—the upper surfaces of which are flat and the under surfaces arched. Finally, they unite to form a coherent and generally a light greyish-yellow, glutinous film, which may extend to the walls of the glass vessel, forming a complete ring. Such a perfect film-formation only occurs after the primary fermentation is at an end. If the flask is shaken, fragments of the film are detached and *sink to the bottom*; and in this way a complete layer may gradually collect at the bottom, whilst the film is continually renewed, and assumes a mottled appearance owing to the fresher portions being thin and dark, whilst the older parts are thick and light.

The conditions under which a film can be formed are a *free, still surface*, with direct access of air; and a vigorous film-formation presupposes an abundant supply of air. The function of film-formation is thus subject to the same conditions as obtain in the case of endogenous spore-formation.

Simultaneously with the formation of a film, a decoloration of the wort takes place, the latter turning to a pale yellow colour This reaction takes place most quickly at higher

temperatures, and occurs most markedly with those species which give rise to the most vigorous film-formation.

The preliminary cultivation of the cells is the same as that previously described (spore-formation). The liquid is then decanted from the growth obtained, and fresh sterilised wort is added; the mixture of yeast and wort is agitated, a drop is transferred—with the usual precautions—to an ordinary flask of about 150 c.cm. capacity, half filled with wort, and a piece of filter-paper is then tied over its mouth. HANSEN exposed flasks treated in this way to different temperatures, and determined:

1. The limits of temperature for the formation of films;
2. The approximate length of time required for their formation at different temperatures; and
3. The microscopic appearance of the growths at different temperatures.

The main point in these investigations of the six species previously mentioned is to determine *the microscopic appearance of the films of these species*, formed *at the same temperature*; and here again, if we may regard the results from a different point of view to that adopted in the last section, we have a complete investigation of the relation between the influential factors and the forms produced, demonstrating that we are dealing with so many perfectly distinct types or species.

The examination of the films was made, unless otherwise stated, when they had so far developed that they were just visible to the naked eye.

A glance at the illustrations representing these film-growths (see description of the species) will show that *their general character is different from that of the sedimentary forms.* For instance, the sedimentary form of *Sacch. cerevisiæ I.* is oval or spherical, whilst in the film, elongated cells quickly appear, and the growth gradually assumes an appearance perfectly distinct from that of the sedimentary yeast.

If we compare the film-formations of the six species, we find that the films developed at the higher temperatures offer very few points of difference which would be of value in their examination; *Sacch. cerevisiæ I.* and *Sacch. ellipsoideus II.* being alone distinguishable from the remainder. It is quite

otherwise, however, when *young films developed at a temperature of* 13° to 15° C. are examined. The two species, *Sacch. Pastorianus II.* and *Sacch. Pastorianus III.*—which are both top-fermentation yeasts, and which in the ordinary cultures cannot be distinguished from each other with certainty by the form of their cells—exhibit in this case entirely different forms of growth; and an equally striking difference is found between the otherwise similar species *Sacch. ellipsoideus I.* and *II.*

An examination of the *limits of temperature* for the formation of films shows that for *Sacch. cerevisiæ I.* and *Sacch. ellipsoideus I.*, these lie approximately within 38° and 5°–6° C.; the limits for the three *Pastorianus* species are 34° and 3° C.; *Sacch. ellipsoideus II.* has the same lower limit as the last species, but its maximum temperature is 38° to 40° C.

The time limits, when compared with those previously given for ascospore-formation, show that in both cases the development takes place more slowly at low than at higher temperatures; at temperatures near to the minimum and maximum limits only a very slight and imperfect film-formation is ever obtained.

At temperatures above 13° C. *the film of Sacch. ellipsoideus II. develops so rapidly and vigorously* that flasks containing this yeast can be recognised by this alone. Thus, at 22° to 23° C. the film had completely covered the surface at the end of six to twelve days, whilst in the case of the other five species a period three times as long was required for the formation of films, and these were generally more feebly developed. This species and *Sacch. Pastorianus III.* also develop a vigorous film with comparative rapidity at the ordinary room temperature, the other species being left far behind.

We have said that the film-formations have different maximum temperatures. This is related to the fact *that the maximum temperature for budding is not the same for the different species.* It was proved that *budding and fermentation can take place at temperatures at which film-formation no longer occurs.* Thus, in the case of *Sacch. cerevisiæ I.*, *Sacch. ellipsoideus I.* and *Sacch. ellipsoideus II.*, HANSEN still observed a vigorous fermentation and budding at 38° to 40° C., and at 34° C. in

the case of the three species of the group *Sacch. Pastorianus*. A relationship is thus shown to exist between the influence of temperature on budding and fermentation on the one hand, and film-formation on the other.

In brewers' low-fermentation yeasts, and in some wild yeasts, WILL observed round and oval cells, having a *thick membrane* and containing a number of small *oil-drops* (Fig. 44)

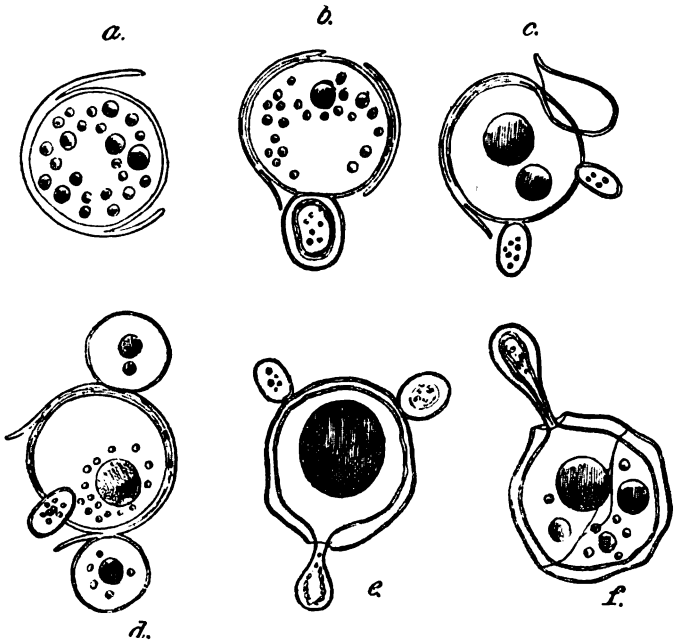

FIG. 44.—Durative cells (after WILL). The outer layer of the membrane is partly or completely detached. *a*, *b*, in wort; *c-f*, in mineral nutrient solution.

These occurred in the rings of yeast and in the small surface patches preceding true film-formation. If treated with concentrated hydrochloric acid, the membrane splits into two layers. In cultures, especially in artificial nutrient liquids, the outer layer of this membrane gradually detaches itself; sometimes in such a way that the outer layer is not torn, so that it appears as if the cell contained another cell. The cell contents are coloured green or brown by concentrated sulphuric acid. The glycogen reaction with iodine has been occasionally observed

in these cells. They appear to play a certain part in the life economy of the growth, *as durative cells*, for these cells are

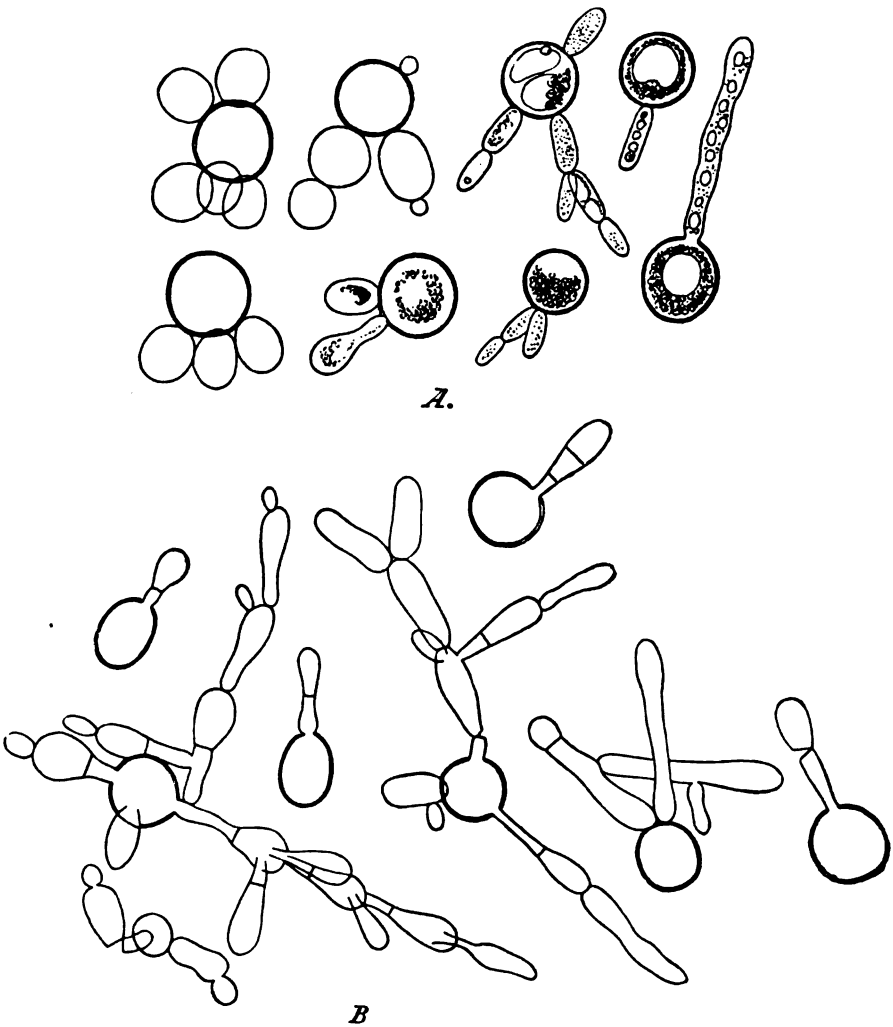

FIG. 45.—Durative cells (after WILL). *A*, mode of germination most frequently found; *B*, durative cells, having produced club or sausage-shaped daughter cells, with transverse walls.

sometimes found alive in old growths after most of the other individuals have perished. In artificial nutrient solutions

containing mineral salts, sugar, and asparagine, with an addition of citric or tartaric acid, such durative cells occur also in the bottom-growth. From these cells germinate globular or oblong yeast-cells, either singly or in large number (Fig. 45*A*). Especially in older cultures of the durative cells that have formed in mineral nutrient solution there frequently spring up club-shaped cells with transverse-wall formations, which phenomenon may recur in derived growths (Fig. 45*B*). During germination on a solid nutrient medium, WILL also observed a splitting-up of these transverse walls (Fig, 45*B*).

(*d*) **The Temperature Limits for the Saccharomycetes.**—Just as the influence of temperature on the development of spores and films varies with the different species, so it has been shown by HANSEN'S investigations (1883) that both spores and vegetative cells of different species possess unequal powers of resistance to hot water. In this respect the spores have a greater resisting power than the vegetative cells.

In experiments of this nature, as in the cases previously discussed, the condition of the cells has a most marked effect on the results; they are especially influenced by age. Thus, it was found that the cells of *Sacch. ellipsoideus II.*, which had been cultivated in wort for two days at a temperature of 27° C., were killed in five minutes when heated to 56° C. in sterilised distilled water, whilst cells of a similar culture 2½ months old were able, under identical conditions, to withstand five minutes' heating to 60° C. without being killed.

Ripe spores of the same species, which had been developed at a temperature of 17° to 18° C., and, in the course of eight days at the same temperature, had partially dried up, withstood a temperature of 62° C. for five minutes, but were killed at 66° C.

In the case of *Sacch. cerevisiæ I.* the vegetative cells are, under similar conditions, killed by five minutes' heating at 54° C., whilst at 62° C. the spores are killed.

An interesting grouping of HANSEN'S six species with reference to a fixed temperature is also exhibited when they are cultivated in wort under conditions favourable to film-formation (see above). When, for instance, a temperature of 36° to 38° C. is employed for the development, the three

Pastorianus species will die in the course of eleven days, whilst *Sacch. cerevisiæ I.* and the two ellipsoid species will still be living. From this result it is evident that the statement formerly accepted that top-fermentation yeasts can develop at higher temperatures than bottom yeasts is incorrect.

KAYSER'S more recent experiments confirm these results, and also show that yeasts can resist a considerably higher temperature in a dry state than in the presence of moisture. For instance, a pale ale yeast was killed when exposed for five minutes in a moist condition to a temperature of 60° to 65° C., but when dry, it withstood a temperature of 95° to 105° C.; in the case of a wine yeast (St. Emilion) the temperatures were 55° to 60° C. and 105° to 110° C. The resisting power of the spores was 10° to 20° higher.

Vegetative cells which had developed from the heated spores exhibited a somewhat greater power of resistance than normal vegetative cells. This increased resistive power was, however, not transmitted further, and, on cultivation in beer-wort, disappeared even in the second generation.

(*e*) **Cultivation on a Solid Nutritive Medium.**—HANSEN discovered distinct characteristics for several species of the *Saccharomycetes* by suitable cultivation on a solid nutritive medium. For this purpose he employed small flasks containing wort, to which about 5·5 per cent. of gelatine had been added, the flasks being closed by means of cotton-wool plugs. When these flasks are inoculated with the six known species (*Sacch. cerevisiæ I., Sacch. Pastorianus I., II., III., Sacch. ellipsoideus I., II.*), and set aside at a temperature of 25° C., the growths which develop show such macroscopic differences, in the course of eleven to fourteen days, that four groups may be more or less sharply distinguished. *Sacch. ellipsoideus I.* stands alone, for its growth exhibits a characteristic net-like structure on the surface, which enables this species to be distinguished by the unaided eye from the other five species. When gelatine with yeast-water is employed for such cultures and the experiment conducted at 15° C., *Sacch. Pastorianus II.* yields growths, after the lapse of sixteen days, the edges of which are comparatively smooth, whilst the growths obtained from *Sacch. Pastorianus III.* are distinctly

hairy at the edges. A microscopical examination shows that in this case the two species are also distinguishable morphologically. This is not by any means always the case with cultures in solid media; in fact, the differences are often less marked under such conditions than when nutritive liquids are employed.

For the *Mycoderma species* and *Sacch. membranæfaciens*, HANSEN has discovered a characteristic behaviour in wort-gelatine, in which they form shield-like colonies readily distinguishable from those of the *Saccharomycetes*.

In this connection we may mention HANSEN'S observation that some species, *e.g.*, *Sacch. Marxianus* and *Sacch. Ludwigii*, can develop a *mycelium* when grown in a solid medium, while others are unable to do so.

In the case of certain cultivated yeasts, P. LINDNER has noted distinct differences in their growths on gelatine.

WILL and others have shown that the characteristics exhibited by cultures on nutritive gelatine are often very variable.

ADERHOLD, during an examination of gelatine-growths of German ellipsoid wine yeasts, found that in puncture-cultures and in drop-cultures two types were distinguishable, one of which showed colonies with funnel-shaped depressions and with marked concentric lines, whilst the other showed conical growths with indistinct concentric structure, but very prominent radial streaks.

A great number of yeast species render nutrient gelatine liquid. This was proved by the author in 1890 with regard to brewers' high-fermentation yeasts. More recently, similar observations have been made with regard to various other yeast species by WILL, WEHMER, FISCHER, and others.

(*f*) **The Behaviour of the Saccharomycetes and Similar Fungi towards the Carbohydrates and other Constituents of the Nutritive Liquids. Diseases in Beer.**—The first striking proof of the fact that *Saccharomyces* species may produce very different reactions in the nutritive liquid, was obtained by means of *pure cultures of the yeasts* prepared in the Carlsberg laboratory, afterwards in the author's, and later on in many other laboratories, and *which were subsequently introduced in practice*. There are breweries in which a con-

siderable number of different species of yeast have been tried on a large scale and under identical conditions, and where the attenuation, taste, odour, time of clarifying, and permanence as regards yeast turbidity, etc., etc., have been found to differ for each individual species.

HANSEN'S epoch-making work on the *disease-yeasts* (1883) have shown, from another point of view, the marked differences amongst the *Saccharomyces* species in their action on the nutritive liquid; he discovered groups of the so-called wild yeasts, which *bring about detrimental changes in beer*, whilst others were found to be *harmless*. Amongst the former, again, there are some which communicate a *bitter taste* and *disagreeable odour* to the beer (*Sacch. Pastorianus I.*) without as a rule producing turbidity; whilst others only fully develop their activity in a late stage of the secondary fermentation, and cause the *beer to become turbid* (*Sacch. Pastorianus III.* and *Sacch. ellipsoideus II.*). The latter effect is due to the abundant yeast deposit formed in a comparatively short time after the finished beer has been drawn off, which rises at the slightest movement of the liquid. It is only when these species—*Sacch. Pastorianus I.*, *Sacch. Pastorianus III.*, and *Sacch. ellipsoideus II.*—are introduced into wort *at the commencement of fermentation* that they are able to induce disease. The addition of disease-yeast to beer in the store casks or to drawn beer has no appreciable effect; the inoculation of bottled beer with *Sacch. ellipsoideus II.* will only take effect when the beer has been very strongly infected. The main result of his work is the proof that the danger of infection *lies in the pitching-yeast*. These diseases have led to very great difficulties and have caused considerable losses in breweries. HANSEN'S observations on the disease-yeasts have been confirmed and extended by the author, GRŒNLUND, WILL, LASCHÉ, KOKOSINSKY, KRIEGER, WINDISCH, and P. LINDNER. Wild yeasts can also bring about disturbing effects in top-fermentation breweries. For instance, the so-called "summer-cloud" of Australian beers is caused, according to DE BAVAY, by a wild *Saccharomyces* species. This organism causes turbidity and imparts an acid, bitter taste to the beer. In English high-fermentation beers the author found yeasts of the *Saccharomyces anomalus* type which

produced turbidity: in weakly fermented Danish high-fermentation beers there occurred *Torula* species with the same properties.

PICHI has recently detected disease-yeasts in wine.

Just as mould-fungi exhibit a different behaviour *towards the various carbohydrates* (see Penicillium, Mucor, Monilia), so the different *Saccharomycetes* and allied fungi have been shown by HANSEN'S comprehensive investigations to exhibit *pronounced characteristics* in the same direction. In addition to the true *Saccharomycetes* we shall review *Mycoderma cerevisiæ, Sacch. apiculatus*, the *Torula* forms, and *Monilia.*

HANSEN studied the behaviour of a large number of yeasts *towards the four carbohydrates—saccharose* (*cane-sugar*), *maltose, lactose, and dextrose.*

His six species of Saccharomycetes (*Sacch. cerevisiæ I., Sacch. Pastorianus I., II.*, and *III., Sacch. ellipsoideus I.* and *II.*) behave as follows:—They all develop invertase; they convert cane-sugar into invert-sugar, which they then ferment; they ferment maltose and dextrose, but not lactose. All the bottom-yeasts used in practice show the same behaviour towards these four sugars.

Sacch. Marxianus, Sacch. Ludwigii, and *Sacch. exiguus* do not ferment maltose and lactose; they invert saccharose and ferment nutritive solutions of invert-sugar and dextrose.[1]

Sacch. membranæfaciens and *Mycoderma cerevisiæ* can neither invert nor ferment these four carbohydrates.[2]

Sacch. apiculatus does not invert saccharose, and of the four sugars mentioned, it only ferments dextrose. It therefore only induces a feeble alcoholic fermentation in beer-wort.

Amongst the *Torula forms* examined by HANSEN there are many which do not secrete invertase, do not ferment maltose, and only yield about 1 per cent. of alcohol (by volume) in beer-wort. Other species invert saccharose. In nutritive

[1] According to EMIL FISCHER, maltose is not fermented directly, but is first inverted by a particular enzyme. Thus, most known species of *Saccharomycetes* contain both the enzyme inverting cane-sugar and that inverting maltose. In the three species, *S. Marxianus, S. Ludwigii*, and *S. exiguus*, on the other hand, only the enzyme which inverts cane-sugar is found.

[2] According to LASCHÉ'S experiments, some species of *Mycoderma* present in beer are capable of inducing alcoholic fermentation.

dextrose solutions the different species induce a more or less vigorous fermentation.

Monilia candida, although possessing no enzyme soluble in water, ferments saccharose, maltose, and dextrose. It ferments beer-wort, but at the ordinary room temperature it only yields the higher percentages of alcohol, at a much slower rate than the *Saccharomycetes*.

In milk, various budding fungi have been found, all of which are able to decompose milk sugar. GROTENFELT and the writer have described some *Saccharomycetes*; DUCLAUX, ADAMETZ, KAYSER, and BEIJERINCK several *non-Saccharomycetes*. Species fermenting milk sugar have never yet been detected in breweries. FERMI found that certain white and red yeasts are capable of exercising a *diastatic* action. MORRIS arrived at similar results in experiments with pressed yeast.

If we now review all these different properties of the *Saccharomycetes*, we shall see that they fall into two groups:—

I. Those which possess an inverting enzyme, and induce alcoholic fermentation. This group is further sub-divided into

(*a*) those which not only ferment saccharose and dextrose, but also vigorously ferment maltose (the six species first described by HANSEN, and the yeasts employed in the brewing industry);

(*b*) those which ferment saccharose and dextrose, but not maltose (*Sacch. Marxianus, Ludwigii* and *exiguus*).

II. Those which do not possess any inverting enzyme, and do not induce alcoholic fermentation (*Sacch. membranæfaciens*).

The *budding fungi which do not form endospores* (*non-Saccharomycetes*) show the most varied characters with reference to the properties of inversion and fermentation.

I. The great majority do *not ferment maltose*. Many of these induce a more or less vigorous fermentation in solutions of dextrose and invert-sugar. Some (*Torula* forms) invert saccharose, and many possess no invertive ferment (*Mycoderma cerevisiæ*, other *Torula* forms and *Sacch. apiculatus*).

II. Only one species (*Monilia candida*) ferments maltose, dextrose, and saccharose without, however, possessing any inverting enzyme soluble in water.

The *lactose-fermenting Saccharomycetes* and *Torula* occupy a separate position.

From the above it is clear that, as HANSEN asserts, the *Saccharomycetes* cannot be characterised merely as alcoholic ferments.

When we consider the behaviour of these fungi in the fermentation industries, it is at once seen that it is only amongst the *Saccharomycetes* that species occur which rapidly and vigorously ferment maltose. The yeasts for breweries and distilleries must therefore be looked for from among the true *Saccharomycetes*. The fungi excluded from the genus *Saccharomycetes*, of which by far the greater number do not ferment maltose, are scarcely capable of playing any important part in these industries; on the other hand they may be employed in the manufacture of wines from grapes, berries, and fruits, since several of them are able to induce just as vigorous a fermentation in solutions of dextrose and invert-sugar, as the *Saccharomycetes*.

It is, then, of the utmost importance that *a suitable species should be selected*.

These different properties of the various species of budding-fungi are of special importance in analytical chemistry, in cases where solutions containing several different carbohydrates are under examination. In fact HANSEN has expressed the opinion that it will be possible in this way to arrive at a more exact quantitative determination of the different carbohydrates in wort. Several chemists have been recently engaged on this problem, but a true solution has not yet been effected.

The characterisation of the synthetically prepared *isomaltose* furnishes a fine example of the application of biological analysis. As is well known, EMIL FISCHER discovered this sugar species in the products of the reaction of hydrochloric acid on grape sugar, at a low temperature, and it received the name of isomaltose, because it appeared to have a constitution similar to that of maltose. The sugar has not yet been prepared in a state of purity, but is known only in the form of

an osazone. C. J. LINTNER recently announced that he had discovered isomaltose in beer, and he then described the formation of the same sugar through the hydrolysis of starch. BROWN and MORRIS, on the other hand, asserted that isomaltose does not exist among the inversion products of starch, but that the substance described by LINTNER as isomaltose is nothing but maltose contaminated by dextrine-like decomposition products. LINTNER has hitherto not succeeded in adducing fresh evidence in support of his view. Even the existence of FISCHER'S synthetically formed *isomaltose* has been questioned, this substance being also regarded as impure maltose. By a fresh investigation, however, FISCHER succeeded in proving biologically that this sugar is sharply distinguished from maltose by the fact that isomaltose is *neither fermented by fresh yeast nor decomposed by the enzymes of yeast*, and he asserts that it is only possible to differentiate with certainty between the two sugar species in this way.

The different action of the *Saccharomyces* species on the same nutritive liquid (*e.g.* wort or must) under identical conditions, has been further studied by BORGMANN, AMTHOR, and MARX.

According to BORGMANN, the chemical reactions brought about in wort by the two Carlsberg bottom-yeasts, No. 1 and No. 2, show a striking difference. These two species—which had been in use for some time in the fermenting room, and were still practically pure—were employed for pitching two fermenting vessels containing wort from the same brew; the fermentation took place under conditions which enabled a true comparison to be made, and the resulting beer was stored as usual. The differences in the products were especially noticeable in the proportion of free acid. Thus:

	No. 1.	No. 2.
Acid (calculated as lactic acid), -	0·086	0·144 per 100 c.c.
Glycerine, - - - - - -	0·109	0·137 "

As a result of these experiments, BORGMANN points out that the ratio between the alcohol and glycerine in these two beers differs from that previously found in beer, the ratio obtained from previous analysis being:

	Alcohol.	Glycerine.
Maximum, - - - - - - -	100	5·497
Minimum, - - - - - - -	100	4·149

whilst the Carlsberg beers gave the following numbers:

	Alcohol.	Glycerine.
No. 1, - - - - - - -	100	2·63
No. 2, - - - - - - -	100	3·24

It is thus seen that, as BORGMANN suggests, good beer may be produced in which the ratio of glycerine to alcohol is lower than the previously admitted minimum.

A series of *eight different species of Saccharomyces*, and amongst them six "cultivated" yeasts, all in absolutely pure cultures, were examined by AMTHOR with reference to their chemical action on beer-wort. His results again confirmed HANSEN'S principle, that, in practice, a careful selection must always be made. The fermentations were conducted in Pasteur flasks of one litre capacity under identical conditions, and in two series, the first of which corresponded to the primary fermentation in the brewery, and the second series to the secondary fermentation. The alcohol, extract, specific gravity, attenuation, glycerine, nitrogen, reducing substance and the degree of colour, were determined in the fermented worts. The tables show palpable differences in the work accomplished by the different species. The percentage of alcohol (by volume) varied within the limits 4·34 and 6·02 (3·55 to 5·94 at the end of the primary fermentation), the extract from 8·27 to 11·23 (8·49 to 12·61 at end of primary fermentation), the attenuation from 36·7 to 53·3 (28·8 to 52·1 at end of primary fermentation); the percentage of glycerine showed very striking differences and fluctuated between 0·08 and 0·15; and likewise the amounts of nitrogen and reducing substance, and to some extent even the degree of colour, showed considerable differences.

HIEPE drew some interesting parallels between the behaviour of a number of culture yeasts and wild yeasts towards the sugars. For this purpose he instituted *fermentations in cane sugar solutions* to which yeast water had been added. He took out the first sample five minutes after the fermentation had been induced, and then fresh samples every day, till the fermentation had subsided. In each sample the amount of (1) inverted cane-sugar, (2) fermented extract, (3) fermented dextrose, and (4) fermented lævulose, was determined.

In these four respects well-marked, specific differences manifested themselves. Thus, in the course of five minutes an English high-fermentation yeast had inverted 1·95 per cent. sugar, whilst a low-fermentation yeast from the author's collection had inverted 58·85 per cent. A complete inversion of the cane-sugar, in two brewers' low-fermentation yeasts, took place in the course of about 24 hours, whilst in the case of *Sacch. exiguus* this reaction required 11 days; with regard to the other species the duration lay between these two limits. The detailed tables given by HIEPE show that the fermentation of the entire amount of extract and also of the two sugar species mentioned takes place successively according to a scale peculiar to each individual species. A cursory glance at the numerous particulars of the experiments further shows that the fermentation of dextrose as a rule begins much more vigorously than that of lævulose; but, whilst the fermentation of the former sugar species reaches the maximum on the second day, that of lævulose does not evince its highest activity till later, in some species even as late as the fifth day; by slow degrees the proportionate amounts of sugar fermented approach each other, and finally the two sugar species disappear simultaneously.

With regard to the *amount of acid* produced in the nutrient liquid, the yeast species also behave differently. Thus PRIOR examined, from this point of view, the fermentation products of a number of brewery yeasts and wild yeasts in hopped wort, and found that the amounts of acid formed varied from 4·7 to 10 c.c. of decinormal caustic soda solution per 100 c.c. of fermented wort; the fixed organic acids varied from 2·1 to 5·4 c.c., the volatile organic acids from 2·1 to 5·8 c.c. The evidence supplied by this work shows that, in culture yeasts, the amounts of fixed organic acids usually exceed those of volatile acids, whereas in HANSEN'S wild yeast species (*Sacch. Pastorianus I., II.*, and *III.*, and *S. ellipsoideus I.* and *II.*) the reverse is the case, the volatile acids surpassing the fixed ones, very considerably in the case of *Sacch. Pastorianus I.*

A large number of *Saccharomycetes* occurring in *must*—absolutely pure cultures of which were prepared by HANSEN'S method—were investigated by MARX (1888), both from a

botanical point of view and with reference to their chemical action on the nutritive liquid. The time required for spore-formation was very different for the different species; the number of spore-forming cells and the number of spores in individual cells also exhibited striking and constant differences. In connection with this, it is remarkable that the pure cultivated species show distinct differences in fermentative power, in the production of those volatile substances which impart a special bouquet to wine, and finally in their power of resistance to different acids and to high temperatures. As marked differences in taste are produced by not a few species, MARX is justified in emphasising the practical importance of such investigations, since it may thus become possible, by the addition of yeasts of known properties to wine-must, to produce wines having definite character as regards taste, etc.

More recently AMTHOR has also investigated a number of absolutely pure cultures of wine yeasts, and has detected typical differences with regard to spore-formation, the time of the fermentation, and in the chemical composition of the wines produced. Similar results have also been obtained by JACQUEMIN, ROMMIER, MARTINAND, and RIETSCH, in France; MUELLER-THURGAU, in Switzerland; NATHAN and WORTMANN, in Germany; MACH and PORTELE, in Austria; FORTI and PICHI, in Italy; some of the comparative experiments conducted by these authors having been carried out on a large scale.

The most thorough and extensive investigations into the different behaviour of wine yeasts towards must are due to J. WORTMANN. He states, as the general upshot of his investigations, that the differences in the activity of the divers types of genuine wine yeast are sometimes so great that they can be detected merely through the chemical analysis of the fermentation products; in other cases, however, they are of such a kind that we can only convince ourselves directly of their presence by means of our senses (smell and taste). Every type of yeast shows some individual peculiarity more or less characteristic of itself in the *action it exerts on any must regardless of its origin or variety.*

The *number* of the *yeast-cells which are formed* in a given

must, apart from the amount of nutrient ingredients existing in the must, depends on the specific power of multiplying of the yeast-type used; on the other hand, it is in itself independent of the origin or composition of the must. In any given must, whether it be an excellent or an indifferent nutrient medium for the wine yeast, one yeast type will multiply more freely than another.

An extensive comparison of the *amount of extract* contained in a number of wines fermented with three different yeast species showed that in the same must the "Würzburg yeast" consumed the smallest quantity of extract; next came the "Johannisberger," whilst the "Ahrweiler yeast" used up the largest amount of extract, and, accordingly, left the smallest residue in the wine.

The specific activity of wine yeasts manifests itself particularly in the formation of *glycerine*. These three species were compared in a large number of musts of different origin, and, generally speaking, the Würzburg yeast formed more glycerine than the other two; of these the Johannisberger yeast was superior to the Ahrweiler, which, as stated above, was distinguished as the one fermenting the extract most satisfactorily. The difference here shown to exist between the chemical behaviour of these species was made still more pronounced by the fact that the "Würzburg yeast" had multiplied least.

Both the amount of *nitrogen* and of *ash* proved to be different in wines fermented with the three species of yeast.

The amount of *acid* was highest in the wines fermented with Würzburg yeast and practically equal for the other two species.

Finally, in accurate comparative experiments, a large number of species proved to differ widely with respect to the amount of *alcohol* produced in the liquid; those yeasts having the shortest time of fermentation yielded the smallest amounts of alcohol, and conversely.

The question, whether the formation of special "*bouquet*" *substances* in the individual type or species of wine yeast is constant, or dependent on the character of the must, a question of great technical importance, was answered in the affirmative by WORTMANN; for he has demonstrated the possibility (con-

firmed by the writer's own experience), of imparting a special bouquet to wine, and thus of essentially improving its taste, and, consequently, enhancing its value in all those cases in which indifferent musts with no prominent qualities are fermented with specially-chosen pure yeasts. This also holds good when grape wine yeasts are used with fruit and berry must.[1]

If the must itself contains a pronounced flavour, an added yeast will not, of course, have a decided effect on the bouquet, but its adoption will be justified by the greatly enhanced purity and regularity of the fermentation. (See further on the subject, Chapter VI.)

KAYSER compared the chemical properties of several types of wine yeast, and found the formation of volatile acids at higher temperatures differed for each species. Thus the quantity of these acids increased in one species, but decreased in another, at a higher temperature.

FORTI, basing his conclusions on comparative experiments with wine yeast, has called attention to the existence of typical differences in the fermentative power of the species, in their power of resistance towards higher temperatures, and in the quantity and quality of the nutrient liquid they demand. According to his view there is a well-marked distinction, in the character of the fermentation produced by the yeasts of the principal, or "vehement," fermentation on the one hand, and those of the secondary or quiet fermentation on the other.

The numerous investigations carried out continuously since 1884, in the author's laboratory, with pure cultures of alcoholic yeasts, as used in the various branches of the fermentation industry, have furnished ample opportunities of collating experience relating to the extremely varied chemical activity of the species and to their respective powers of *retaining their peculiarities intact* during preservation, a matter of importance

[1] Considerable scientific and practical interest attaches to the much-debated question, whether the amounts of *alcohol* and *glycerine produced, bear any certain proportion to each other.* WORTMANN'S experiments, all conducted with absolutely pure cultures, established the fact that this proportion is subject to great fluctuations, even in one and the same type of yeast. Thus, contrary to former assertions, no one definite proportion can be established as normal, *e.g.*, in deciding whether a wine is adulterated or not.

to each individual department of our industry. Numerous instances were met with in which even slightly noticeable characters, manifested through taste or smell, remain inherent after several years' preservation of the growth and a suitably renewed development of the culture under favourable circumstances.

(*g*) **Variations in the species of the Saccharomycetes.**—HANSEN'S numerous investigations have proved that the *Saccharomycetes* are affected in various ways by external influences. From the results recorded in the previous sections, we are perfectly justified in saying that there are a number of species, not only of the so-called wild yeasts (species which were formerly described under the general names *Sacch. Pastorianus*, *Sacch. ellipsoideus*, etc.), but also of well-characterised top- and bottom-yeasts, which are employed in practice. It is a point of great practical interest that species cultivated in beer-wort, the cultivation of which has been uninterruptedly continued for several years, have shown only the slightest changes, or none at all. While HANSEN was arriving at these results, he discovered that it was possible, by suitable treatment, to produce variations in different directions; and that in this respect, also, the individual peculiarities of the cells in an absolutely pure culture may assert themselves. Some of these changes are only temporary, and disappear under suitable treatment, while the species reassumes its original character. Others are more deeply seated, and it is then only under especially favourable conditions that the culture can be deprived of its newly-acquired properties. In certain cases it was found impossible, even after years of methodical treatment, to re-convert a growth into its original state.

1. As we have stated, the data regarding the time required for *the appearance of the first indications of spore-formation* in the six species previously described, are subject to the condition that the growth has been previously cultivated in wort for 24 hours at a temperature of 25° C. Simultaneously with the publication of his temperature curves (1883), HANSEN found that cultures which had been grown in wort at the above temperature, but for *two* days instead of one, developed spores

more slowly and more sparingly than usual. If, however, such cultures are *subsequently treated in the manner first described, the culture again assumes its normal condition.* We have here, therefore, an example of a very feebly-rooted variation.

2. In a gelatine culture of "*Carlsberg bottom-yeast No.* 1" both *oval and elongated, sausage-shaped cells* are often found, so that, according to REESS, the presence of two species must be assumed. If colonies of each kind are separately introduced into flasks containing wort, growths are again obtained which consist partly of egg-shaped and partly of "*Pastorianus*" cells. HANSEN'S experiments showed that when these latter cultures were repeatedly re-cultivated in fresh flasks the cells partially retained their sausage-shape for a lengthened period. When such a culture was introduced into a yeast-propagating apparatus, the growth obtained from it still showed an admixture of these cells; these disappeared, however, after the yeast from the propagating apparatus had been introduced into an ordinary fermenting vessel. In this case, therefore, the variation is more strongly rooted, and only disappears after the yeast has been propagated through a series of fermentations.

As early as 1883 HANSEN showed that the limits drawn by REESS in the characterisation of his species do not exist, because in the case of each pure culture we are able to develop the different species as defined by REESS. Shape and size of the yeast-cell are very variable. The sausage-shape (*Sacch. Pastorianus Reess*) readily passes into the oval shape (*Sacch. cerevisiæ ellipsoideus exiguus Reess*), and conversely.

Another example of a similar character is that of a species of *Sacch. cerevisiæ* (a bottom-yeast) which, after a lengthened and difficult development, was subsequently cultivated in wort at a temperature of about 27° C. when the cells obtained exhibited their *ordinary appearance*; when cultivated at 7·5° C., however, *grouped colonies with mycelium-like branches* were obtained. This is an interesting example of the influence of temperature on the form of yeast-cells.

3. As an example of a much *more deeply-rooted change* in the nature of the cells, HANSEN'S observations on *Sacch. Ludwigii* may be mentioned. When single individuals, taken

from an absolutely pure culture, are again separately cultivated as pure cultures, it is possible to obtain growths which exhibit *great differences in their power of forming spores.* By a methodical choice of single cells HANSEN succeeded in obtaining growths which, under the recognised conditions, completely failed to yield spores; on the other hand, he found that, when starting from the same original growth, a yeast speck which had sprung from a spore-yielding cell was chosen and further developed, a growth was obtained which was forthwith capable of yielding an abundance of spores. By such methodical selection, three varieties were separated from this species, one of which was characterised by its high capacity of forming spores; in the second this property had nearly disappeared, whilst the third did not form spores at all. *After numerous cultivations in wort, the third form reverted, but only slowly, to its original condition, in which it was able to form spores; when it was cultivated in a solution of* 10 *per cent. dextrose in yeast-water, however, this property was immediately re-acquired.*

In other species, varieties which have lost their power of spore-formation completely, or in part, may make their appearance, without any assignable cause, both on liquid and on solid nutrient media. In some cases (*e.g. Sacch. Ludwigii*) that power is restored if *dextrose* is added to the nutrient liquid.

If a pure culture of brewery yeast is developed in a *wort which has not been aërated* after sterilisation, it generally loses its normal "*breaking*" and *clarifying* properties, under brewery conditions, and this to a degree dependent on the species. These new variations must often be cultivated through very many generations in ordinary brewery wort before regaining the original qualities of the species. As aëration brings about changes in the chemical composition of the wort, it is evident that this effect on the plasma is due to such modifications in the nutrient medium.

As an additional instance of the effect of the chemical composition of wort in producing new varieties, we may mention the observation, due also to HANSEN, that *Sacch. Pastorianus I.*, which imparts an unpleasant taste and smell to beer-wort, is apt, if preserved in *an aqueous solution of cane-sugar*, to lose this power for a time.

A similar proof of a variation in brewers' low-fermentation yeast, brought about by the composition of the nutrient liquid, was furnished by SEYFFERT, who found that it was possible, in the case of a good selected type which, after long use in breweries, had lost its good properties with regard to clarification (breaking), to restore it to its original condition by treatment with a lime salt. Gypsum was added either to the wort, the brewery water, or the steeping-vat, and from wort prepared in this way wort-gelatine was concocted, in which the degenerated yeast growth was sown for fresh pure-cultivation. On development of the colonies in small flasks, these new growths showed true "breaking" and the power of adhering to the bottom of the flask, and the qualities thus regained were retained during the use of this yeast in practice.

Another example of physiological transformation is the following: The three species described under the group *Saccharomyces Pastorianus* form under certain conditions a *dough-like sediment* similar to those of the other *Saccharomycetes*; under other conditions, however, a *film-like, wrinkled,* or *caseous sediment* consisting of small lumps (PASTEUR'S *levûre caséeuse*), that is to say, sediment of very different appearance, and yet produced by the same species. In the latter case, the fermenting wort also assumes a very characteristic appearance, and, contrary to what ordinarily occurs, remains bright throughout the fermentation, so that yeast flakes may be observed rising to the surface and sinking again to the bottom. If this curious sedimentary yeast is repeatedly cultivated by new fermentations in wort, it can be again transformed into the dough-like condition.

Finally, we also find a transitory physiological transformation in the *film-formations of the Saccharomycetes.*

4. At the beginning of the year 1889, HANSEN published[1] the results of a series of experiments which were undertaken with the hope of discovering *the conditions causing variation, and of experimentally bringing about the formation of new races, and if possible new species.* He has recently published additional work on the subject.

[1] Centralbl. f. Bakt. u. Parasitenk., Bd. V., p. 665, 1889.

(A) He found in the case of the typical *Saccharomyces* species, that when their cells were cultivated *in aërated wort at a temperature above the maximum for their spore-formation and near the maximum for their vegetative growth*, they were affected in such a manner that *they lost their power of forming spores and films*, and the same applies also to the innumerable generations successively formed in new cultures under the most varied conditions. He succeeded also in bringing about such a transformation by cultivation on solid media.

In some of the species treated in this way, it was also observed that, in wort-cultures, they yielded a more abundant crop of yeast, but a slower fermentation. This was, for instance, the case with Carlsberg low-fermentation yeast No. 2. The newly formed variety attenuated more slowly and weakly than the original species; but at the same time the clarification was better.

RAYMAN and KRUIS have shown that the cells present in films possess the power of oxidising alcohol produced during the fermentation, into carbon dioxide and water. HANSEN'S varieties, while completely losing the power of forming films, are rendered incapable of performing this oxidising action. Thus, while a flask, containing the original species, which had developed a luxuriant film after six months' standing, showed only 1·5 per cent. by volume of alcohol, a parallel flask, which showed no film-formation, contained 5·5 per cent. of alcohol—a quantity equal to that found at the end of the first month.

In another series of experiments HANSEN showed that the action of *higher temperatures* upon the cells *without aëration* was capable of producing radical and lasting alterations of a different kind in the nature of the plasma. When Carlsberg low-fermentation yeast No. 1. was cultivated in wort at 32° C. through eight cultures, each successive culture being inoculated from the preceding one, which had been left undisturbed until the end of the fermentation, a variety was evolved in the ninth culture which, in wort of 14° Balling, to which 10 per cent. of saccharose had been added, formed 1-2 per cent. by volume less alcohol than the original form. The new variety clarified better under brewery conditions, and gave a weaker

attenuation at the end of the primary fermentation. The same, was found to be the case with other species.

(B) HANSEN also succeeded, by cultivation in *nutrient gelatine* in producing new constant varieties or species.

Thus, two varieties of Carlsberg low-fermentation yeast No. 1, each generation of which was transferred to the surface of wort-gelatine, attained a fermentative power superior to that of the original forms.

It was observed in the experiments on spore-transformations brought about by the action of temperature and aëration, that if cells of the successive generations are selected, many had been affected even in the *first* growths under the new conditions; this modification, however, is only temporary in its character; it is only after successive generations have been allowed to develop through continued inoculation under the new conditions that the acquired characters become constant. It appears from this that the transformation does not depend on *temperature* or *aëration* alone, but also on the *nutrition* and *propagation* of the cells.

A comparison of these different factors has, however, shown that they contribute unequally to the result. Both the nutrient liquid and the aëration only bring about vigorous new-formations, and may therefore vary considerably without materially affecting the result. This, however, is not true of temperature; a fluctuation of a few degrees is sufficient to prevent the variations described from coming into existence. Hence, it follows, that *temperature* plays the principal part in these transformations.

No objection can be raised to the view that we are possibly dealing with the formation of new species. We know in fact that the species are not fixed and unchangeable, as was generally assumed in LINNÉ'S time, but that the characters of a species are only constant under certain conditions. The complete elucidation of these important and intricate problems can, however, only be effected by a series of experiments carried on through a long period of time and under a variety of conditions.

As previously stated, these remarkable changes are only brought about by a long-continued and violent interference

with the vital process of the cells; they do not occur so long as development takes place in the normal manner.

The variation of low- and high-fermentation yeast in breweries has occupied the writer's attention for many years, and has led to several researches. These have proved that a "*degeneration*" *of a brewery's yeast* may be brought about by a deviation from the normal of a large number of the individuals of which the growth consists, their acquired properties exercising a disturbing influence on the course of fermentation. In such a case the yeast can be restored to its original condition by selecting "non-degenerate" individuals from a sample of the yeast used in the brewery. This is done in the following manner:—A sufficiently large number of cells are isolated, absolutely pure cultures are developed from these individuals, and a culture is selected which exhibits the required properties.

The variations which yeast undergoes during its use in the factory were further employed by the author with the object of systematically *improving yeast.* For this purpose samples were taken from particularly good fermentations, and from this yeast whole series of cells were isolated. Among the growths descended from these, such were selected as were found to possess the required properties in a marked degree. Methodical work in this direction, carried on during several years with one and the same type of yeast, has led to decidedly favourable results.

An example of the persistence with which *Saccharomyces* cells retain, under normal conditions, the property of spore-formation, is met with in breweries and distilleries. We have here species of yeasts which have lived through hundreds of years, and have developed an infinite number of generations under conditions which, as a rule, have not permitted the exercise of this function, and yet the power of forming spores has been persistently retained.

(*h*) **Gelatinous Formation secreted by the Budding-fungi.**—Under certain, as yet undetermined, conditions, the colonies produced by the budding of yeast-cells can form irregular agglomerations which sink to the bottom more quickly than the single yeast-cells (breaking and clarifying

in the brewery). This behaviour is undoubtedly related to a phase in the development of yeast-cells which HANSEN discovered in 1884. He found that not only the *Saccharomycetes* but also other budding-fungi are able to secrete a *gelatinous network*, which can be seen as threads or plates, and in which the cells lie embedded (Fig. 46, A, B). If, for

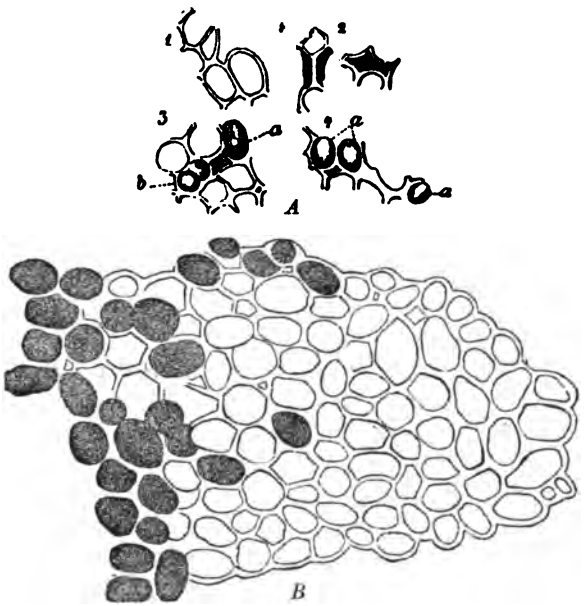

FIG. 46.—Yeast cells with gelatinous network (after HANSEN): *A*, network obtained by partial drying; 1, portion formed of threads, from which the cells have become detached; 2 and 3 show that the network can also form complete walls; such a formation is seen between *a* and *b*; *a* is a vegetative cell, *b* is a cell with two spores; 4, shows three cells, *a*, embedded in the network. *B*, network with yeast cells, the latter stained by methyl-violet; the network is not stained. Some of the yeast cells are still in the meshes, but most have detached themselves.

example, a moderately thick paste of brewery yeast is placed in a glass and allowed to remain under cover in such a way that it slowly dries, and then a trace of this yeast is mixed with water, the network can be clearly seen (Fig. 46, A). The formation also occurs in the gypsum block and gelatine cultures. The author has frequently observed this remarkable formation, after HANSEN had called attention to its nature, in the yeast samples which are sent to his laboratory in filter-

paper enclosed in envelopes.[1] HANSEN also found it in the film-formations of nearly all species. An ordinary microscopic examination of the pitching-yeast in a brewery does not show this formation; with the help of staining, however, its presence can be readily detected (Fig. B). When the yeast is repeatedly washed, it is no longer possible to detect the network by staining; but if the water is removed, and the yeast set aside for a time and then suitably treated, the gelatinous masses can be readily seen. By varying the conditions of nourishment of the cells, the development can be promoted or retarded, and the chemical composition modified. The whole behaviour suggests the zooglœa-formation of bacteria.

STRUCTURE AND GENERAL NATURE OF THE YEAST-CELL.

The usual microscopic appearance of a yeast-cell, as it most frequently occurs in a fermenting liquid, is a spherical or oval figure which gives rise to one or more buds by the swelling out of its wall, these detaching themselves sooner or later from the mother cell. The membrane may vary somewhat in the different stages of development of the cell, but rarely to a noticeable degree. It is otherwise, however, with the contents of the cell. The contents present the simplest appearance when the cell is observed in its most vigorous state of growth. The cell-contents then consist of clear homogeneous plasma. As the processes of multiplication and fermentation continue, different bodies make their appearance in this plasma; certain clear portions filled with sap (vacuoles), other larger and smaller particles, some of which can be shown to be fat globules, whilst others appear to be of a similar nature to the plasma. These granules have been minutely described by RAUM and EISENSCHITZ. The granular appearance of the plasma increases with the further development of the cell, and at a very advanced stage of the fermentation, when the cell

[1] This method of preserving a sample of yeast for a time is very convenient. A small piece of filter-paper is rapidly passed through a flame several times, a few drops of yeast are poured onto it; it is then folded up, and afterwards wrapped in several layers of paper which have been similarly treated.

has almost come to a state of rest, the plasma may be reduced to a thin layer on the inside of the wall, whilst a large vacuole occupies the remaining space, containing numerous large and small grains, many of a fatty nature. If such cells are again brought into a fermentable liquid, they exhibit a highly characteristic appearance during the period which precedes the macroscopic phenomena of fermentation. The grains disappear, and numerous fine plasma-threads appear in the clear cell-sap, and gradually surround the vacuoles; finally these disappear, and the cell is once more filled with clear homogeneous plasma.

If the yeast is cultivated in beer-wort with 10 per cent. cane-sugar, or in a decoction of sugar-beet, or in certain artificial nutrient liquids, at 30° C., the plasma of the cells is coloured brownish-violet by iodine; the coloration disappears on heating, but reappears at low temperatures. This reaction has been held to be a proof of the presence of *glycogen*—a reserve substance (a carbon compound) which occurs in animals and also, it is supposed, in various fungi, where it apparently plays a part analogous to that of starch in green plants.

As in most vegetable cells, a *cell-nucleus* (first discovered by SCHMITZ) is also found in the yeast-cell, and its presence can be proved by staining with osmic acid or with picric acid and hæmatoxylin. According to HANSEN this cell-nucleus is either spherical or disc-shaped. In old film-formations of *Saccharomycetes*, he found cells which distinctly showed the nucleus without any treatment.—JANSSENS, DANGEARD, and BUSCALIONI observed the partition of the cell-nucleus in the budding and in the spore-formation of the *Saccharomycetes*.

CLASSIFICATION OF THE SACCHAROMYCETES.

The majority of *Saccharomycetes* are only known as *budding-fungi with endogenous spore-formation.* In the minority, a mould-stage is also known, which bears a certain resemblance to *Dematium*, *Oïdium*, or *Monilia.*[1]

[1] (Compare page 124). Experimental evidence of the descent of *Saccharomycetes* from other fungi has not as yet been adduced. BREFELD showed that many *Ustilagineae* (smut-fungi), *Basidiomycetes*, and other fungi may enter

In a distinct group vegetative propagation takes place only by *division through transverse walls.*

In most species the germinating *spores* grow into *budding cells*; in some the spore puts out a *germinating thread.*

The *Saccharomyces* cells in one and the same species occur in different forms.

In the cells *nuclei* are present, but they can only be observed, as a rule, after special treatment.

The cells secrete a gelatinous net-work, which under favourable conditions may develop freely.

The greater number of the species induce alcoholic fermentation.

As was remarked above, the names *Sacch. cerevisiæ, ellipsoideus, Pastorianus,* etc., are now used in a sense widely different from that originally attached to them by REESS. HANSEN'S work clearly showed that the distinctive characters of the *species* cannot, as supposed by REESS, be foretold by the size and shape of the cells *in themselves.* A species which under certain conditions may occur in forms, denoted by REESS as *Saccharomyces cerevisiæ, i.e.,* with large oval cells, may, under other conditions, develop a growth which according to REESS is to be described as *Sacch. ellipsoideus,* and, conversely, the same species which under certain circumstances are to be described as *S. cerevisiæ* or *S. ellipsoideus* must under other conditions be designated as *S. Pastorianus.* Thus, shape and size of the cells can only be used as distinctive characters of the species if, in conjunction, it is stated under what particular conditions of cultivation the growth has developed. As, however, under *identical* conditions of growth there are a large number of species which, according to the shape of the cells, would all have to be described as *Sacch. ellipsoideus, Sacch. cerevisiæ,* etc., it follows that REESS'S names, if used in the sense under discussion, would form the names of *groups of*

upon a budding-fungus stage. Similar phenomena were previously observed by BAIL, DE BARY, REESS, ZOPF, and others. As BREFELD did not show whether these forms possess the power of endogenous spore-formation characteristic of *Saccharomycetes,* nor whether they are able to display any marked fermentative activity, his assertions that they are equivalent to *Saccharomycetes* are destitute of foundation.

species, these species being again distinguished by a series of special characters. The following systematic descriptions are chiefly based upon the results of HANSEN'S experimental researches. According to this investigator, it is considered probable, for many reasons, that the oval form of yeast-cell is the original one, and that the different, more or less marked Pastorianus growths have developed from it.

SACCHAROMYCES CEREVISIÆ I. (HANSEN).[1] (Figs. 47-49.)

This and the five following species (*Sacch. Pastorianus I., II.*, and *III.*, *Sacch. ellipsoideus I.* and *II.*), all develop invertase and maltase; with these they effect the hydrolysis of

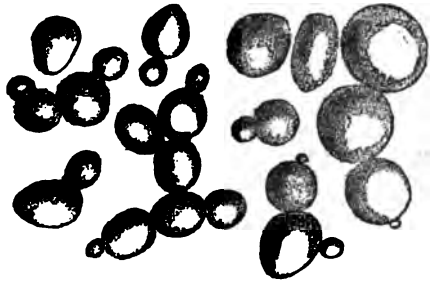

FIG. 47.—Saccharomyces cerevisiæ I. (HANSEN). Cell-forms of young sedimentary yeast (after HANSEN).

saccharose and maltose to invert sugar, and ferment the latter. They produce a vigorous fermentation in dextrose solutions, and likewise in maltose solutions, especially when a nutrient liquid such as yeast-water is added. All are vigorous alcoholic ferments, which in the course of fourteen days, at the ordinary room-temperature, readily produce 4 to 6 per cent. (by volume) of alcohol in beer-wort. They are unable to ferment lactose.

Saccharomyces cerevisiæ I. is an old English top-fermentation yeast, which is employed in breweries in London and Edinburgh.

The young growth of sedimentary yeast (Fig. 47) developed in wort, consists essentially of large round and oval cells; really elongated cells do not occur under these conditions.

[1] This top-fermentation yeast must not be confused with HANSEN'S Carlsberg bottom-yeast No. 1.

Ascospore-formation (Figs. 37-39, 43, 1):—[1]

At	37·5° C.	no ascospores are developed.	
36–37°	the first indications are seen after	29	hours.
35	„	25	„
33·5	„	23	„
30	„	20	„
25	„	23	„
23	„	27	„
17·5	„	50	„
16·5	„	65	„
11–12	„	10	days.
9	no ascospores are developed.		

Spores strongly refractive to light. Wall of spores very distinct. Size of spores 2·5–6 μ.

FIG. 48.—Saccharomyces cerevisiæ I. (HANSEN). Film-forms at 15—6° C. (after HANSEN).

Film-formation :—

At	38° C.	no film-formation occurs.	
33–34°	feebly-developed film-specks are seen after	9–18	days.
26–28	„	7–11	„
20–22	„	7–10	„
13–15	(Fig. 48)	15–30	„
6– 7	(Fig. 48)	2– 3	months.
5	no film-formation occurs.		

[1] The preparatory treatment of a *Saccharomyces* species for these investigations must be made in the following manner:—After the cells have been cultivated for some time in ordinary wort (14° Ball.) at the ordinary room-temperature, the vigorous young cells obtained are introduced into fresh wort, in which they are allowed to develop for about twenty-four hours at 25° to 27° C. This growth is used for the gypsum-block culture.

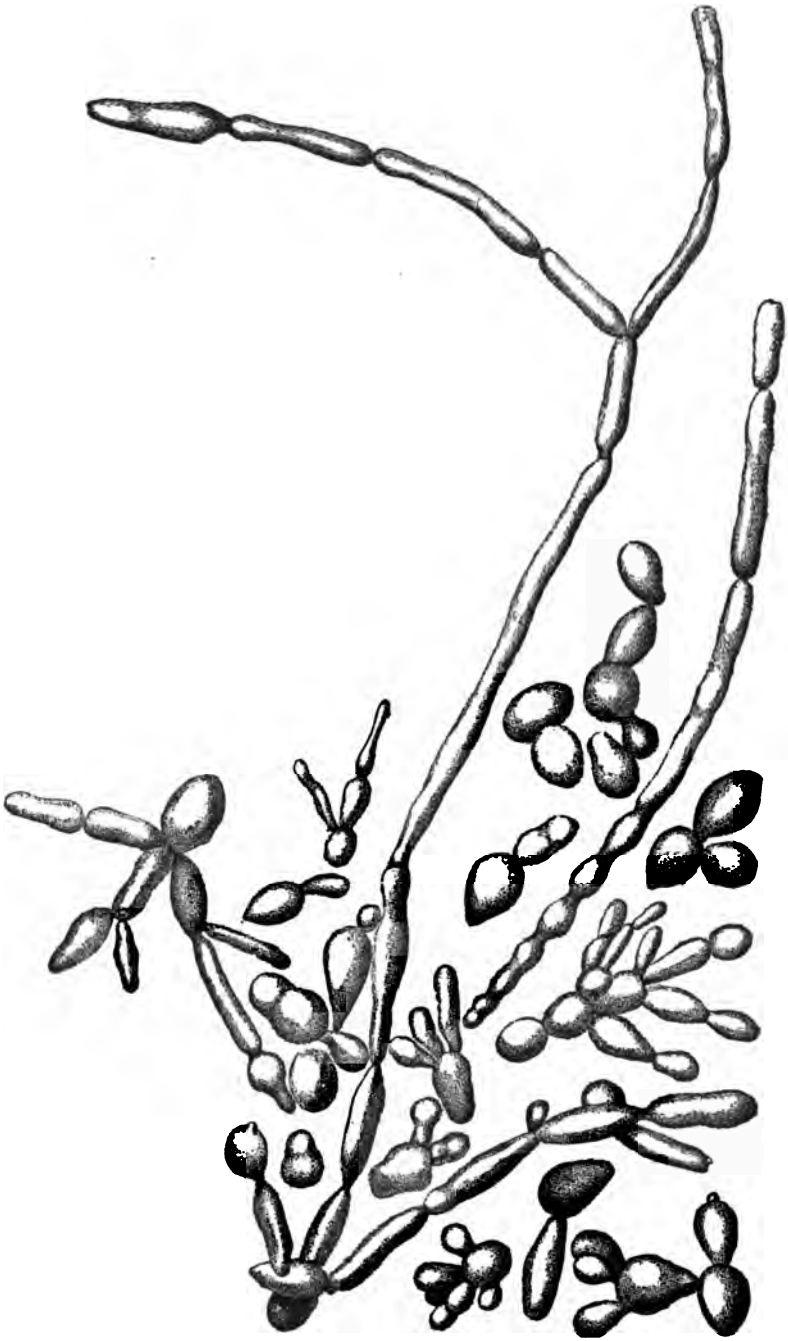

FIG. 49.—Sacch. cerevisiæ I. (HANSEN). Cell-forms in old cultures of films (after HANSEN).

Microscopic appearance of the cells in the films :—

At 20–34° C.: Colonies frequent; sausage-shaped and curiously formed cells occur.

At 15–6° C. (Fig. 49): The greater number of the cells resemble the original cells; isolated cells of different form occur.

In old cultures of films all forms occur, including greatly elongated mycelium-like cells (Fig. 49).

SACCHAROMYCES PASTORIANUS I. (HANSEN). (Figs. 50, 51.)

Bottom-fermentation yeast.

Sedimentary forms grown in wort:—Mainly elongated,

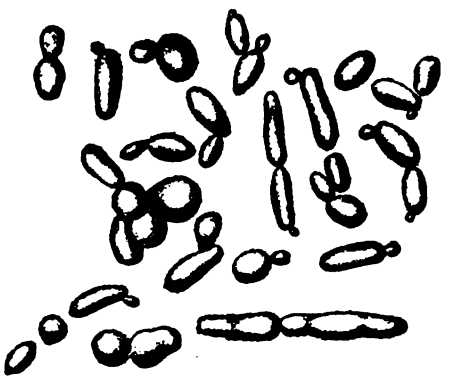

FIG. 50.—Saccharomyces Pastorianus I. (HANSEN). Cell-forms of young sedimentary yeast (after HANSEN).

sausage-shaped cells, but also large and small, round and oval cells (Fig. 50).

It frequently occurs in the air of fermenting-rooms. It imparts to beer a disagreeable *bitter* taste and unpleasant odour; it may also produce turbidity, and interfere with the clarification of beer in the fermenting vessel.

According to investigations of MACH and PORTELE, this species may also be successfully used in wine fermentation.

Ascospore-formation (Fig. 43, 2):—

At	31·5° C.	no ascospores are developed.	
	29·5–30·5°	the first indications are seen after	30 hours.
	29	„ „	27 „
	27·5	„ „	24 „
	23·5	„ „	26 „
	18	„ „	35 „
	15	„ „	50 „
	10	„ „	89 „
	8·5	„ „	5 days.
	7	„ „	7 „
	3–4	„ „	14 „
	0·5	no ascospores are developed.	

Size of spores 1·5–5 μ.

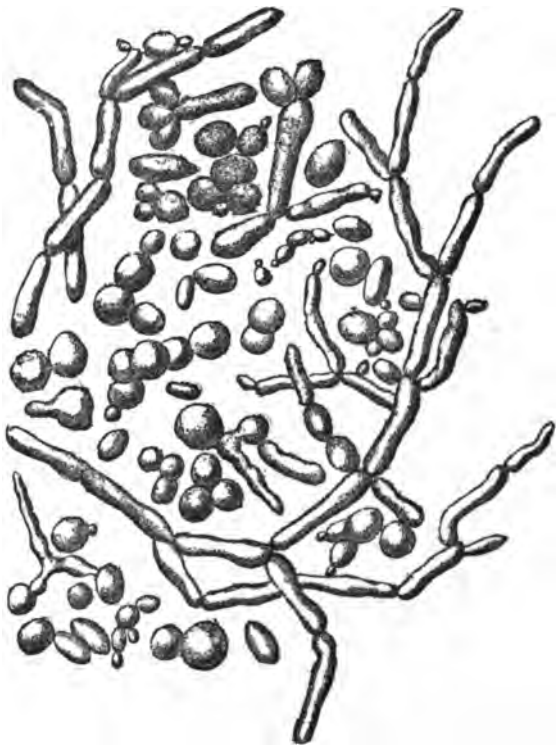

FIG. 51.—Saccharomyces Pastorianus I. (HANSEN). Film-forms at 13–15° C., from Holm's drawing in HANSEN's Memoir.

Film-formation :—

At 34° C. no film-formation occurs.

26–28° feebly-developed film-specks are seen after - - - - 7–10 days.

20–22 „ „ 8–15 „

13–15 } (Fig. 51) { „ 15–30 „

6– 7 } „ 1– 2 months.

3– 5 „ „ 5– 6 „

like Fig. 51, but without the large colonies.

2– 3 no film-formation occurs.

Microscopic appearance of the cells in the films :—

At 20–28° C. almost the same forms occur as in the sedimentary yeast.

At **13–15**° C. strongly-developed mycelium-like colonies of very elongated, sausage-shaped cells (Fig. 51) are moderately frequent.

In *old cultures of films* the cells are smaller than in the sediment; very irregular and sometimes almost thread-like cells are found.

SACCHAROMYCES PASTORIANUS II. (HANSEN). (Figs. 52, 53).

Feeble top-fermentation yeast.

Sedimentary forms grown in wort :—Mainly elongated,

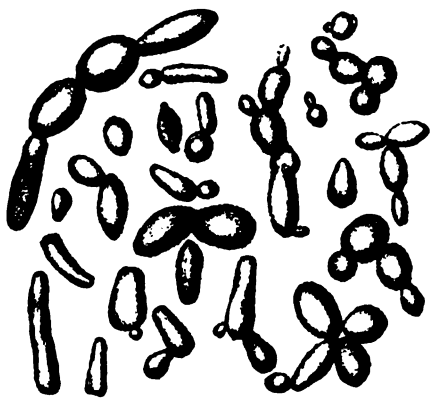

FIG. 52.—Saccharomyces Pastorianus II. (HANSEN). Cell-forms of young sedimentary yeast (after HANSEN).

sausage-shaped cells, but also large and small, oval and round cells (Fig. 52).

It frequently occurred in HANSEN'S analyses of air in the brewery; it appears to belong to the species which do not acuse diseases in beer. Ascospore-formation (Fig. 43, 3):—

At 29° C. no ascospores are developed.

27–28°	the first indications are seen after	34	hours.
25	„ „	25	„
23	„ „	27	„
17	„ „	36	„
15	„ „	48	„
11·5	„ „	77	„
7	„ „	7	days.
3–4	„ „	17	„

0·5 no ascospores are developed.

Size of the spores 2–5 μ.

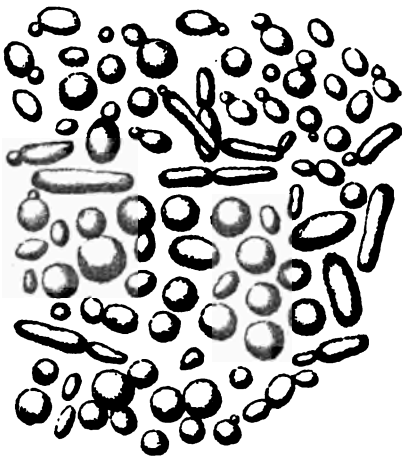

FIG. 53.—Saccharomyces Pastorianus II. (HANSEN). Film-forms at 15—8° C. (after Holm's drawing in HANSEN'S Memoir).

Film-formation:—

At 34° C. no film-formation occurs.

26–28°	feebly-developed film-specks are seen after	- - -	7–10 days.
20–22	„	„	8–15 „
13–15	(Fig. 53)	„	10–25 „
6– 7	(Fig. 53)	„	1– 2 months.
3– 5	(Fig. 53)	„	5– 6 „
2– 3	no film-formation occurs.		

Microscopic appearance of the cells in the films:—

At 20–28° C.: Almost the same forms as in the sedimentary yeast; also irregular sausage-shaped cells.

At 15–3° C.: Mostly oval and round cells.

In *old cultures of films* the cells are smaller than in the sediment; very irregular and sometimes almost thread-like cells are found.

Streak cultures of this species in *gelatine yeast-water* give growths with comparatively *smooth edges* after sixteen days at 15° C., and in this respect it also differs from the following species.

SACCHAROMYCES PASTORIANUS III. (HANSEN). (Figs. 54, 55.)

Top-fermentation yeast.

Sedimentary forms grown in wort:—Mostly elongated, sausage-shaped, but also large and small, oval and round cells (Fig. 54).

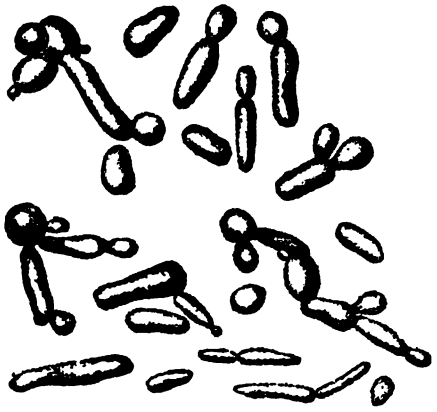

FIG. 54.—Saccharomyces Pastorianus III. (HANSEN). Cell-forms of young sedimentary yeast (after HANSEN).

It was separated from a bottom-fermentation beer which showed *yeast-turbidity*, and has been proved by HANSEN to be one of the species which produce this disease. Recent experiments of HANSEN show that this disease-yeast possesses another peculiar property; namely, when the fermenting wort has an

opalescent appearance, the addition of *Sacch. Pastorianus III.* will in certain cases effect a clarification.

According to investigations made by the author, a strong infection of low-fermentation yeast with this species may in certain cases effect an excellent clarification and good "breaking" in both the fermentation vessel and in cask.

Ascospore-formation (Fig. 43, 4):—

At	29° C.	no ascospores are developed.	
	27–28	the first indications are seen after	35 hours.
	26·5	„ „	30 „
	25	„ „	28 „
	22	„ „	29 „
	17	„ „	44 „
	16	„ „	53 „
	10·5	„ „	7 days.
	8·5	„ „	9 „
	4	no ascospores are developed.	

Size of the spores 2–5 μ.

Film-formation:—

At	34° C.	no film-formation occurs.	
	26–28	feebly-developed film-specks are seen after - - -	7–10 days.
	20–22	„ „	9–12 „
	13–15	(Fig. 55) „	10–20 „
	6– 7	(Fig. 55) „	1– 2 months.
	3– 5	(Fig. 55) „	5– 6 „
	2– 3	no film-formation occurs.	

Microscopic appearance of the cells in the films:—

At 20–28° C.: Almost the same forms as in the sedimentary yeast.

At **15–3°** C.: Strongly-developed colonies of elongated, sausage-shaped or thread-like cells, which closely resemble a mycelium in appearance (Fig. 55).

In *old cultures of films*, the cells have the same forms as at 3–15° C., but are often still thinner and more thread-like.

Streak cultures of this species in *gelatine yeast-water*, after

sixteen days at 15° C., give growths with distinctly *hairy edges.*[1]

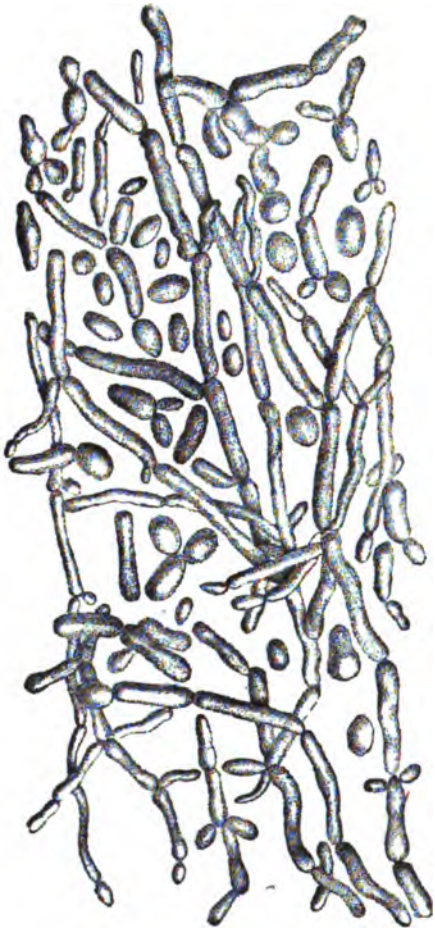

FIG. 55.—Saccharomyces Pastorianus III. (HANSEN). Film-forms at 15—3° C. (after HANSEN).

[1] To the *Pastorianus* group also belongs the "*Logos yeast*," described by VAN LAER, a rapidly clarifying low-fermentation yeast, indigenous to Brazil. During fermentation its cells collect together in bulky, ramified agglomerations, which settle on the bottom and walls of the fermentation-vessels. The *Logos yeast* ferments very slowly, but produces, by degrees, very high percentages of alchohol. According to ROTHENBACH, this species is able to ferment about half the total quantity of a diastase-dextrine, prepared according to LINTNER'S directions, but may be distinguished from the *Sacch. Pombe*, described below, by fermenting species of dextrine not attacked by the latter.

SACCHAROMYCES ELLIPSOIDEUS I. (HANSEN). (Figs. 56, 57.)

Bottom-fermentation yeast.

Sedimentary forms grown in wort:—Mostly oval and round cells; sausage-shaped cells are rare (Fig. 56).

Occurs on the surface of *ripe grapes*.

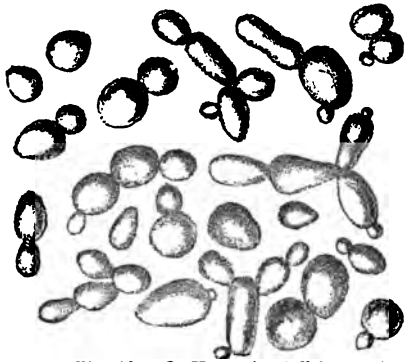

FIG. 56.—Saccharomyces ellipsoideus I. (HANSEN). Cell-forms of young sedimentary yeast (after HANSEN).

Ascospore-formation (Fig. 43, 5):—

At	32·5° C.	no ascopores are developed.	
	30·5–31·5°	the first indications are seen after	36 hours.
	29·5	„ „	23 „
	25	„ „	21 „
	18	„ „	33 „
	15	„ „	45 „
	10·5	„ „	4½ days.
	7·5	„ „	11 „
	4	no ascospores are developed.	

Size of the spores 2–4 μ.

Film-formation:—

At	38° C.	no film-formation occurs.	
	33–34	feebly-developed film-specks are seen after - - -	8–12 days.
	26–28	„ „	9–16 „
	20–22	„ „	10–17 „
	13–15	(Fig. 57) „	15–30 „
	6– 7	„ „	2– 3 months.
	5	no film-formation occurs.	

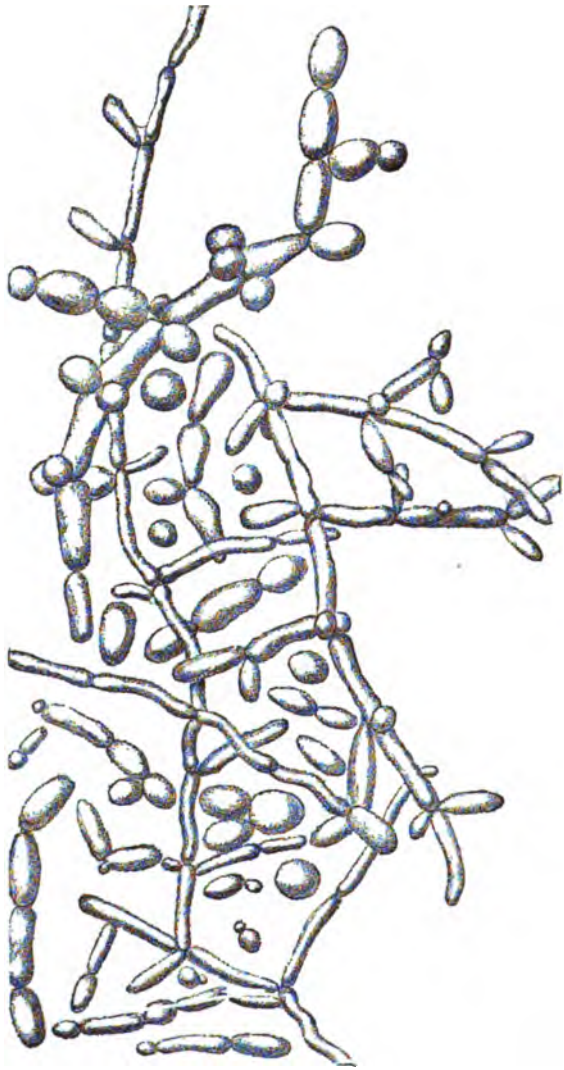

FIG. 57.—Saccharomyces ellipsoideus I. (HANSEN). Film-forms at 13—15° C. (from Holm's drawing in HANSEN's Memoir).

Microscopic appearance of the cells in the films :—

At 20–34° C. and 6–7° C., the cells are smaller and more sausage-shaped than in the sedimentary yeast.

At **13–15°** C., freely-branched and strongly-developed colonies of long or short sausage-shaped cells, often with verticillated branches (Fig. 57).

In *old cultures of films*, the cell forms are the same as at 13–15° C.

Streak cultures of this species in *wort-gelatine* (wort with the addition of about 5·5 per cent. of gelatine), in the course of eleven to fourteen days at 25° C., give—in contradistinction to the other five species—a characteristic *net-like structure*, by means of which it can be distinguished by the naked eye from other species.

SACCHAROMYCES ELLIPSOIDEUS II. (HANSEN). (Figs. 58, 59.)

Usually a bottom-fermentation yeast.

Sedimentary forms grown in wort :—Mostly oval and round cells; sausage-shaped cells are rare (Fig. 58).

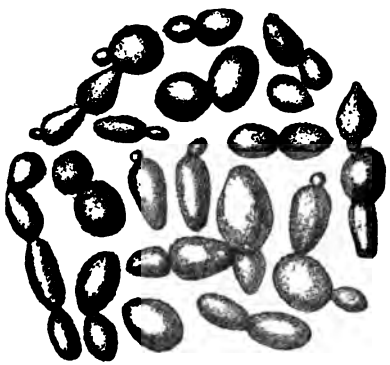

FIG. 58.—Saccharomyces ellipsoideus II. (HANSEN). Cell-forms of young sedimentary yeast (after HANSEN).

It was separated from beers which showed yeast-turbidity; is a species which *causes yeast-turbidity*, and has been shown by HANSEN'S experiments to be more dangerous than *Sacch. Pastorianus III.*

Ascospore-formation (Fig. 43, 6):—

At	35° C.	no ascospores are developed.	
	33–34°	the first indications are seen	after 31 hours.
	33	„ „	27 „
	31·5	„ „	23 „
	29	„ „	22 „
	25	„ „	27 „
	18	„ „	42 „
	11	„ „	5½ days.
	8	„ „	9 „
	4	no ascospores are developed.	

Size of spores 2–5 μ.

Film-formation:—

At	40° C.	no film-formation occurs.	
	36–38	feebly-developed film-specks are seen after - - - -	8–12 days.
	33–34	„ „	3– 4 „
	26–28	(Fig. 59) „	4– 5 „
	20–22	„	4– 6 „
	13–15	„	8–10 „
	6– 7	„	1– 2 months.
	3– 5	„	5– 6 „
	2– 3	no film-formation occurs.	

Microscopic appearance of the cells in the films:—

At all temperatures, the same forms as in the sediment; at

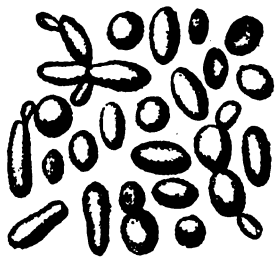

FIG. 59.—Saccharomyces ellipsoideus II. (HANSEN). Film-forms at 28—8° (after HANSEN).

and below 15° C., the cells are only slightly more elongated (Fig. 59).

In *old cultures of films* there are colonies of long and short sausage-shaped cells, often with verticillated branches.

Related to this are two ellipsoid species, described by WILL, which are also *disease-yeasts*. One of these, a bottom-fermentation yeast, gives colonies in wort-gelatine, which, when young, form—whether on the surface or embedded in the gelatine—a network with large meshes; afterwards they grow denser in the middle, with irregularly-fringed edges; sometimes, however, compact colonies with regular outline are formed under the same conditions. The maximum temperature for spore-formation is 39° C.; at the optimum temperature (34° C.), the first indications of spores are seen after eleven hours. The lower limit for spore-formation is 4° to 5° C. The vegetative cells are killed when heated in sterilised wort for half an hour at 70° C. The temperature limits for film-formation are 41° and 4° C. In old films, more especially, numerously-branched clusters are found, consisting of much elongated cells. This species imparts a *rough bitter after-taste* to beer and also causes *turbidity*.

The second ellipsoid species which was obtained from a beer showing yeast-turbidity, gives colonies in wort-gelatine, some of which are sharply defined, whilst in others the outline is indistinct. The temperature limits for spore-formation are 32° and 0·5° C., the optimum temperature being 24° C. The temperature limit for the vitality of the vegetative cells in wort is 70° C. In old films very numerously-branched clusters occur. Besides causing *yeast-turbidity*, this species also imparts a sweetish, *disagreeable, aromatic taste* to beer, and a *bitter astringent after-taste*. The yeast sediment always has a dark colour.

SACCHAROMYCES ILICIS (GROENLUND),

which was found on the fruit of *Ilex Aquifolium*, is a bottom-fermentation yeast, consisting mainly of spherical cells. The temperature limits for spore-formation are 8° and 38° C. The spores have no vacuoles. In the films, slightly-elongated cells are found. Streak cultures on gelatine have a floury, but otherwise a variable, appearance. This species, grown in wort, imparts a disagreeable, bitter taste. According to SCHJERNING,

it contains invertase, and induces alcoholic fermentation in solutions of saccharose, dextrose, and maltose. In ordinary beer-wort it can produce about 2·8 per cent. of alcohol (by volume).

SACCHAROMYCES AQUIFOLII (GROENLUND)

was also found on the fruit of *Ilex Aquifolium*. It is a top-fermentation yeast, and consists of large round cells. The temperature limits for spore-formation are 8° and 31° C.; the spores contain vacuoles. In the films, spherical and egg-shaped cells alone occur. Streak cultures in gelatine vary in appearance, some being glossy and some floury. This species imparts to wort a disagreeable, sweet taste, with a bitter after-taste. It inverts saccharose and induces alcoholic fermentation in solutions of saccharose, dextrose, and maltose. In ordinary beer-wort it can produce about 3·7 per cent. of alcohol (by volume).

SACCHAROMYCES PYRIFORMIS (MARSHALL WARD)

(see Ginger-beer Plant, page 84).

SACCHAROMYCES VORDERMANNI

was discovered in Java by WENT and PRINSEN-GEERLIGS as an essential agent in the manufacture of arrack. It is distinguished by its powerful action as an alcoholic ferment, and yields a fine product, on account of which it is made use of, in pure culture, in that manufacture. The cells are ellipsoid, and form up to four spores. This species ferments dextrose, maltose, and saccharose, and contains invertine.

SACCHAROMYCES MARXIANUS (HANSEN).

This species, which was discovered by MARX on grapes, and described by HANSEN, develops in beer-wort in the form of small oval cells, practically similar to those of *Sacch. exiguus* and *ellipsoideus*. Elongated, sausage-shaped cells, often in colonies, soon appear, however, and if the culture is set aside

for some time, small mould-like particles are formed; some of these swim in the liquid, others settle to the bottom. These particles consist of mycelium-like colonies of practically the same character as the film-formations of the six species previously described; they are also built up of cells, which are readily separated at the point of union. The ascospores are kidney-shaped, spherical, or oval. After cultivation for two to three months in wort contained in two-necked flasks, there were only traces of film-formation with few sausage-shaped and oval cells.

This yeast is one of those species which develop a mycelium under certain conditions of culture on a solid nutritive medium.

In beer-wort it yields only 1 to 1·3 per cent. (by volume) of alcohol, even after long standing. It does not ferment maltose; it inverts saccharose; and in nutritive solutions of the latter, and of dextrose, it yields considerable quantities of alcohol.

The maximum temperature for spore-formation lies between 32° and 34° C., the minimum between 4° and 8° C., the optimum between 22° and 25° C. The growth yields quicker and more abundant spore-formation if cultivated in yeast-water, or wort with 10 per cent. dextrose (KLOECKLER).

In agreement with his theory that maltose is split up by a particular enzyme differing from invertase, and only subsequently fermented, EMIL FISCHER found that an aqueous extract of the pulverised growth of this yeast decomposes saccharose, but not maltose.

SACCHAROMYCES EXIGUUS (REESS), (HANSEN)

develops a growth in wort, the cell-forms of which most closely correspond to the species described by REESS under the above name. It is, however, impossible to determine whether REESS was really dealing with this species, since any *Saccharomyces* species may, under certain conditions, form a preponderating number of similar small cells.

This species only gives scanty spore-formation and weak film-formation, but it yields a well-developed yeast-ring. The cells of the film resemble those of the sedimentary yeast, but short sausage-shaped and small cells are more frequent.

HANSEN found this species in pressed yeast. Its behaviour towards the sugars is similar to that of the last species, though it develops a greater fermentative activity in solutions of saccharose and dextrose. In wort, it yields only small quantities of alcohol. It does not ferment maltose solutions. It inverts saccharose.

Experiments of a practical character, carried out by HANSEN, have shown that this species does not produce any disease in beer, even when present in considerable quantities either at the beginning or end of the primary fermentation, or when it is added after the storage of beer.[1]

Some other species examined by HANSEN can likewise ferment saccharose and dextrose, but not maltose and lactose.

SACCHAROMYCES JOERGENSENII (LASCHÉ)

also belongs to the group of the *Saccharomycetes*, which may be termed *Sacch. exiguus*. The growth consists of small round and oval cells. The optimum temperature for spore-formation is 25° C., the temperature limits being 8° and 30° C. At temperatures above 30° C. the growth dies rapidly. A true film-formation has not been observed; in old cultures only a very feeble yeast ring forms, and this consisted of round and oval cells. In gelatine it yields colonies which resemble those of low-fermentation brewery yeast. Wort-gelatine is slowly liquefied. The streak-culture is dirty grey in appearance, with smooth edges. This species ferments saccharose and dextrose, but not maltose. When it is mixed with cultivated yeasts and grown in malt-wort, it is consequently suppressed. In "temperance beer," according to LASCHÉ'S statement, it produces a strong turbidity.

SACCHAROMYCES MEMBRANÆFACIENS (HANSEN).

This peculiar species, which occupies a special place amongst the *Saccharomycetes*, yields a strongly-developed light grey,

[1] This is of special interest, as *Sacch. exiguus* was formerly regarded as a disease-producing species.

wrinkled film when grown in wort, which very quickly covers the whole surface of the liquid, and consists mainly of sausage-shaped and elongated oval cells; these have strongly-developed vacuoles, and a more or less empty appearance. Separating the colonies is an abundant admixture of air.

The spores are very abundantly developed, not only under the ordinary conditions of cultivation, but also in films. They are irregular in form, and, at the ordinary room-temperature, germinate in a Ranvier chamber after ten to nineteen hours.

On wort-gelatine, the cells form dull grey specks, often with a faint, reddish tinge, which are rounded, flat, wide-spread, and wrinkled. The colonies embedded in the gelatine present, however, a very different appearance. The gelatine is liquefied by this fungus.

This species is incapable of fermenting either saccharose, dextrose, maltose, or lactose; neither does it invert saccharose. It was found in the slimy secretion on the roots of the elm, and shows considerable resemblance to the species *Mycoderma cerevisiæ* and *Mycoderma vini*, but it is a true *Saccharomyces*.

The maximum temperature for spore-formation is 33-33½° C.; the minimum temperature 3-6° C., the optimum lying near 30° C. (17-18 hours). (NIELSEN.)

KOEHLER found this species in very impure well-water. PICHI has described two species which very closely resemble *Sacch. membranæfaciens*.

In the writer's laboratory the species was detected in bright wines.

Other species which readily form a film and produce no fermentation are *Sacch. hyalosporus*, described by LINDNER, with globular spores, forming a tender film on wort; and *Sacch. farinosus*, which forms a white pleated film on wort, making it appear as if it had been sprinkled with flour; the growth consists of long cells, which assume very irregular forms on wort-gelatine.[1]

[1] Another species which also develops very quaint cell-forms upon gelatine, resembling amœbæ, is the *Sacch. Bailii* described by the same author. It brings about slight fermentation phenomena in wort, ferments dextrose and cane-sugar, and forms a mere trace of a film.

SACCHAROMYCES HANSENII (ZOPF)

was discovered by ZOPF among the fungi of cotton-seed flour. It forms very small spherical spores, which usually develop singly, and at most in pairs, in the mother-cell. It does not induce alcoholic fermentation in fermentable nutrient sugar solutions, but, on the other hand, crystals of calcium oxalate have been observed in the sediment. ZOPF found such crystals in nutrient solutions of galactose, grape-sugar, cane-sugar, milk-sugar, maltose, dulcite, glycerine, and mannite.

SACCHAROMYCES LUDWIGII (HANSEN). (Figs. 40, 41 and 60.)

This remarkable species, which was discovered by LUDWIG in the viscous secretion of the living oak, is the only one of the known *Saccharomycetes* which can be recognised solely by means of a microscopic examination. The following description is taken from HANSEN'S investigations. The cells are very variable in size, elliptical, bottle-shaped, sausage- or frequently lemon-shaped. Partition walls may occur in all the complex cell-combinations. The vegetative growths in wort-gelatine are round like those of nearly all *Saccharomycetes*, and are either pale grey, or faintly yellow. In wort, it yields only 1·2 per cent. by volume of alcohol after a long continued fermentation; and this accords with the fact that maltose is not fermented by this species. In dextrose solutions, on the other hand, it yields up to 10 per cent. by volume of alcohol. It inverts saccharose, but does not ferment solutions of lactose and dextrin, neither does it saccharify solutions of starch. It readily develops spores in aqueous solutions of saccharose, in wort-gelatine, in yeast-water, and in wort; in the latter case, even when no film has formed.

It is characteristic of this species that, especially in the case of young spores, *a fusion of germinated spores* often occurs, and *these new formations develop germ-filaments* (*promycelium*), from which new yeast-cells are gradually marked off by sharp transverse septa. At the ends of these cells, buds develop, and these again are marked off by transverse septa.

In old cultures there is often a strong tendency to form *mycelium*, but portions are only exceptionally found the cells of which are firmly united together, showing only slight constric-

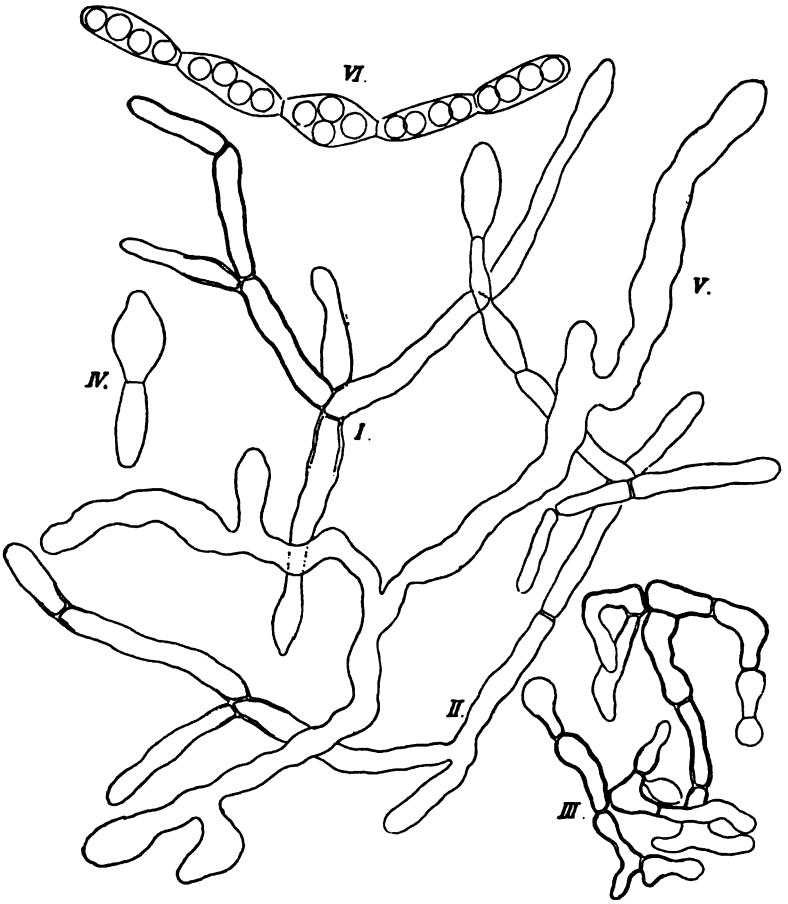

FIG. 60.—Saccharomyces Ludwigii. Old film and mycelium (after HANSEN).

tions; these portions have distinct, straight, transverse walls. Each cell of such a colony can form buds and spores. Amongst them are also found irregular and very large cells with many branches.

It is also characteristic of this species that, when kept in a

solution of saccharose, the cells may die within two years, whilst most of the other *Saccharomycetes* examined may be preserved in this liquid for a much greater length of time.

The maximum temperature for spore-formation is 32-32½° C.; the minimum temperature 3-6° C.; the optimum lies at 30-31° C. (18-20 hours). (NIELSEN.)

Species of this type have frequently been isolated from English and American apple-cider in the writer's laboratory.

Closely allied with *Saccharomyces Ludwigii* are a number of newly-discovered species, in which bud-formation has entirely disappeared, propagation taking place only by division.

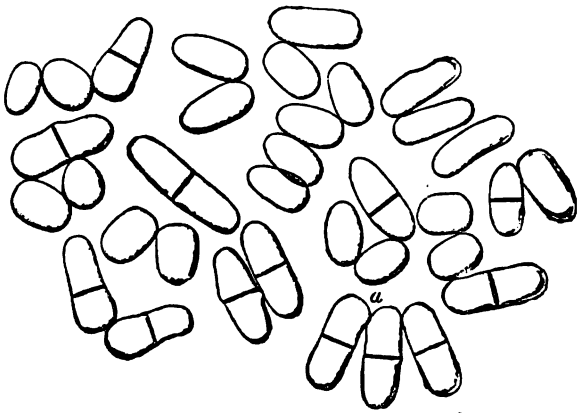

FIG. 61.—Saccharomyces octosporus. Young vegetation after cultivation during 24 hours in beer-wort, at 25° C. (after SCHIOENNING).

Saccharomyces (*Schizosaccharomyces*) *octosporus* (Figs. 61, 62, 63), was discovered by BEIJERINCK on dried currants, and has been more recently examined in the author's laboratory by SCHIOENNING in growths on currants. The propagation takes place in the following manner (Fig. 61): About the middle of the cell a partition wall appears; after this has split up, the two new cells assume a round shape and revolve round a point of the partition-wall, where connection is still maintained, so that at last they lie almost parallel. Finally, they separate entirely from each other, having taken an ellipsoid or oval shape; they then expand in length, and the division begins afresh. But it may

also occur that two cells, still connected throughout the full extent of the partition-wall, grow considerably longer, and form fresh partition-walls, so that the original mother-cell appears divided into four or more cells. Even at the beginning of the fermentation in wort at 25° C., the cells form endospores; but the spore-formation is very inconsiderable, both under ordinary fermentative conditions in wort and also during cultivation on moist gypsum-blocks. This development is much more vigorous on the surface of nutrient gelatines, such as wort-gelatine (Fig. 62), where it forms round waxy colonies with a cavity in the centre. The cells grow shorter and more rounded after developing for some days at the temperature of the room, and the ascus-formation, according to SCHIOENNING'S observations in the Carlsberg laboratory, now takes place as follows (Fig. 62):—

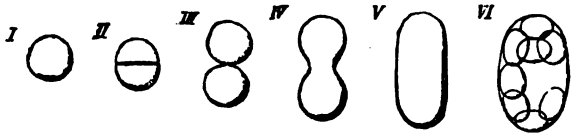

FIG. 62.—Saccharomyces octosporus. Development of the ascus (after SCHIOENNING).

The roundish cell grows longer; a partition-wall appears, which divides, after which the two new cells merely touch or are contiguous at one point. Then they again coalesce (compare the fusions observed by HANSEN in *Sacch. Ludwigii*), and at last form a lengthened, ellipsoidal, hour-glass shaped or irregular cell, which gradually increases in bulk, and within which a varying number of spores (usually eight) are formed. By degrees the wall of the mother-cell dissolves, and the spores now lie embedded in slime, which subsequently disappears. The spores are often oval, and according to LINDNER their membrane is coloured blue by a solution of iodine in potassium iodide.

Both on the vegetative cells and on the asci of the spores very slender lines may sometimes be observed, which form the limit between the older, thicker parts of the cell-wall and the newly-formed, thinner parts. The latter appear after the partition-walls, which now form the terminal walls of the new

cells, have divided, or after fusion, through the ensuing growth of the spore.

On wort no film has been observed; only a slender yeast-ring.

This species ferments maltose and dextrose, but not saccharose; indeed, the last is not even inverted by it. According to

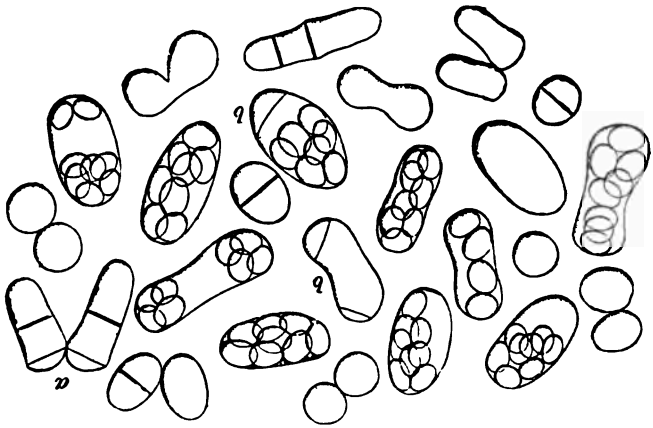

FIG. 63.—Saccharomyces octosporus. Young vegetation in wort-gelatine (after SCHIOENNING).

FISCHER, an aqueous extract of the dried, pulverised growth decomposes maltose, but does not exert any influence upon saccharose.

SACCHAROMYCES COMESII

was described by CAVARA in 1893. It lives as a parasite or saprophyte on the sheaths or pedicles of millet, and, according to CAVARA, forms a mycelium consisting of cylindrical hyphæ with partition-walls; this mycelium produces cylindrical or longish-ellipsoidal conidia 7-9 μ long and 2-3 μ broad, isolated or linked together. The spores are globular, two to four in each cell. Like *Sacch. Ludwigii*, this species is propagated by disjunction and not by bud-formation, and the author has observed that fusion also takes place in the germination of spores.

Saccharomyces (*Schizosaccharomyces*) *Pombe* was discovered by

SAARE and ZEIDLER in millet beer from Africa, and more exactly described by LINDNER. It is closely allied to the previous species; its propagation also takes place by formation of partition-walls and by disjunction; frequently the two new cells remain connected for some time at a single point, upon which they rotate until they form an acute angle to each other. The cells resemble the conidia of Oïdium; but the shape of many of them is suggestive of the manner in which they were derived, one end being rounded, whilst the other is surrounded by a well defined ring-wall, enclosing the newly-formed piece of globular and opalescent membrane. In the cells from one to four spores may occur, which grow in the same way as those of *S. Ludwigii*, viz., by the formation of a germinative thread; no fusion of the promycelium of the spores has been observed.

The growth forms no film on wort. On wort-gelatine it forms a compact finely-furrowed growth.

At its optimum temperature, 30-35° C., this species shows high fermentation phenomena. It is distinguished by the considerable amount of acid formed during the fermentation, and possesses a certain power of resistance in competition with bacteria. In beer-wort it gives rather a vigorous fermentation; it also produces fermentation in dextrose and cane-sugar solutions.

According to investigations by ROTHENBACH, it ferments about half the total amount of a diastase-dextrine prepared according to LINDNER'S directions, leaving acchro-dextrine, which, on addition of alcohol, slowly separates out in sphærocrystals.

As this species is capable of forming very considerable amounts of alcohol, it might be supposed to be available for practical purposes. Experiments made in this direction, however, have not hitherto proved successful.

In the *rum-fermentation of molasses* in the West Indian Islands two different yeast-types are used. In a few districts the common ellipsoidal form predominates; in other districts a mould-like *Saccharomyces*. VORDERMANN and EIJKMANN in Java constantly found, in arrack fermentation of molasses, a fungus which separates new cells through formation of partition-

walls; no spore-formation was observed, and the fungus, according to EIJKMANN, recalls *Hyphomycetes* in its growth-forms. A *Saccharomyces* of similar appearance was discovered by P. GREG, while working in the writer's laboratory, in cane-sugar molasses as used in rum-fermentation in *Jamaica*. J. CH. HOLM and the writer have further investigated this species, which we propose to call

SACCHAROMYCES MELLACEI, N. SP.

(Figs. 64 and 65), and which yielded the following results:—In cylindrical vessels at 25° C. the yeast ferments beer-wort with top-fermentation phenomena, forming a caseous, loose deposit.

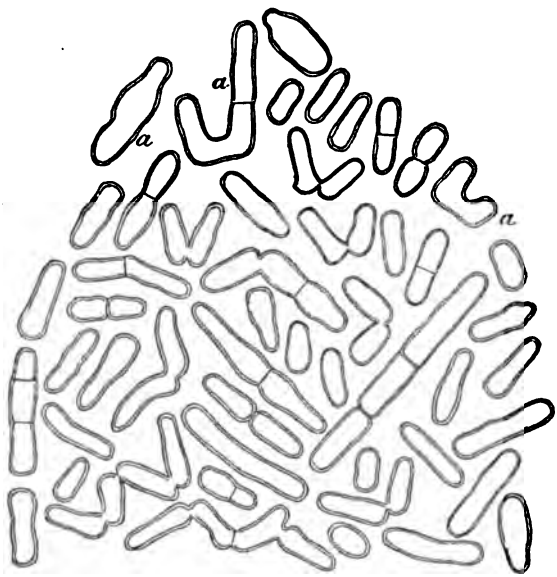

FIG. 64.—Saccharomyces mellacei. Young culture in beer-wort; *a*, cells after cultivation during 8 days. (HOLM.)

During fermentation it develops a pleasant aroma. In wort of 10·5 per cent. Ball it produces about 2½ per cent. by weight of alcohol.

The different forms assumed by the species, recall *Saccharomyces octosporus*, *Sacch. Pombe*, etc. In old cultures very

curious cell-forms (Fig. 64 *a*) occur, which also develop during fermentation. In wort-cultures five months old no film had developed; only a yeast-ring was observed. The liquor is not decolourised by old cultures.

The spores (Fig. 65) are usually oval. They occur in all cell-forms, generally four to a cell; they refract light strongly, and, according to HOLM, they are coloured blue by iodine.

In plate- and streak-cultures, the growths, both on and below the surface, have a sharp cut edge; the cell-forms are similar to those in liquids; cells shaped like the conidia of *Oïdium lactis* frequently occur.

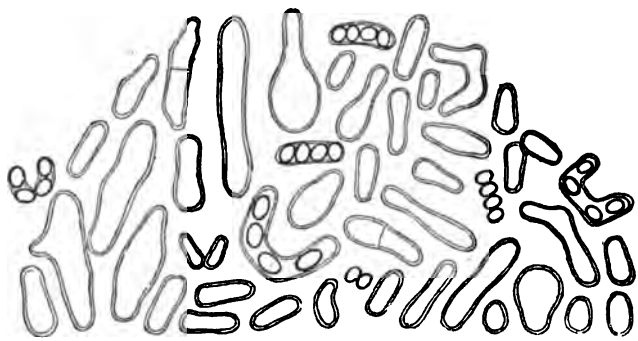

FIG. 65.—Saccharomyces mellacei. Vegetation from the yeast-ring in beer-wort. (HOLM.)

According to investigations made by P. GREG in the author's laboratory, divergencies of a marked and permanent character distinguish the species belonging to this type. Thus some yield malodorous products in the fermenting liquor, others differ greatly in the length of time required to complete the fermentation in one and the same sterilised molasses and under identical conditions. The amount of alcohol produced by these types varied from 6·6 to 7·6 per cent. by volume. The rate of multiplying also differed widely in these species. Further details relating to comparative results in practice are given in papers by GREG and by HART, who has carried out rum-fermentation with ellipsoidal species.

SACCHAROMYCES ACIDI LACTICI (GROTENFELT).

GROTENFELT has described under this name a species of *Saccharomyces* which, when added to sterilised milk, produces a pronounced curdling with formation of acid. On gelatine and agar-agar it forms white porcelain-like colonies, and on potatoes it yields broad, moist patches of a whitish-grey colour, soon turning brown. In puncture cultivations in gelatine short bottle-shaped growths develop from the point of inoculation inwards. The cells are elliptical, 2·0 to 4·35 μ in length, and 1·50 to 2·90 μ in breadth.

When a solution of milk-sugar is inoculated in the presence of calcium carbonate, and the product distilled, alcohol can be detected. In a neutral, 3 per cent. solution of milk-sugar, *Saccharomyces acidi lactici* yielded 0·108 per cent. of acid in eight days.

SACCHAROMYCES FRAGILIS, N. SP. (Figs. 66 and 67.)

While the budding fungi found in kephir, and described by others, do not form spores, a genuine *Saccharomyces* has been

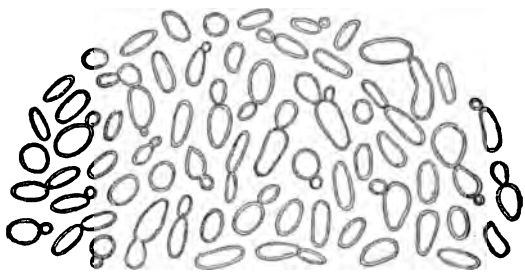

FIG. 66.—Saccharomyces fragilis. Young vegetation in lactose yeast-water. (HOLM.)

discovered in the author's laboratory, which we have called *Saccharomyces fragilis* on account of the feeble power of resistance of the cell-wall.

The growth consists of relatively small, oval, and longish cells (Fig. 66), which refract light feebly and in a peculiar way. At the room temperature, this species behaves as a low-

fermentation yeast. In cultures on gypsum blocks spore-formation begins after 20 hours at 25° C., and in 40 hours not a few free spores may be observed (Fig. 67); at 15° C. the spore-formation takes place in about 40 hours. The *oval* shape of the spores is characteristic of the species. Spores are also formed in growths in fermenting liquids and on gelatines, and in all cases are soon set free. After long standing, the growth forms a thin film, the cell-forms of which deviate comparatively little from those of the deposited yeast. In plate-cultures the surface colonies formed in the course of two or three days at the room-temperature are film-like, while the embedded colonies exhibit thickly haired, mycelium-like borders.

In lactose yeast water (10 per cent), at the room-temperature, this species yielded about 1 per cent. by weight of

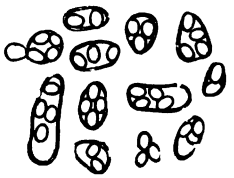

FIG. 67.—Saccharomyces fragilis. Spore-formation. (HOLM.)

alcohol in the course of eight days. In two months as much as 4 per cent. by weight of alcohol was produced. At the same time the formation of acid began. In hopped wort (about 11 per cent. Ball.) it yielded at the room temperature about 1 per cent. by weight of alcohol in ten days.

The optimum temperature for development lies at about 30° C.

According to BAU this species ferments milk-sugar completely, but not melibiose.

SACCHAROMYCES MINOR (ENGEL).

The vegetative cells are completely spherical, measuring up to 6 μ in diameter, and they are united in chains or in groups composed of but few cells (6 to 9). Spore-forming cells are 7 to 8 μ, containing 2 to 4 spores, 3 μ in diameter.

ENGEL considered this organism to be the most active agent in the fermentation of bread.[1]

[1] Decisive experiments on the *essential active factors in the fermentation of bread* have not yet been made. In making white bread, "*pressed-yeast*" is ordinarily used; this consists mainly of alcoholic ferments, and yeast is the only active ferment. In the preparation of black bread, and in some countries also of white bread, so-called *leaven* is employed; this is made by kneading together flour, bran, and water, and allowing the mass to undergo spontaneous fermentation. It contains bacteria in large numbers, and also yeast-like cells, and amongst the latter alcoholic ferments. Antagonistic views have, however, been expressed respecting the relative importance of these different organisms in the fermentation of black bread.

According to CHICANDART (1883) and MARCANO the active ferment is a bacterium. BOUTROUX attributed the fermentation to the activity of both bacteria and budding fungi; he afterwards regarded alcoholic yeast as the chief cause. LAURENT regarded the so-called *Bacillus panificans* as the main cause of the fermentation of bread. DUENNENBERGER'S investigations led to the conclusion that the budding fungi must be looked upon as the only essential organisms of fermentation in bread. The rising of dough is caused in the first place by the carbon dioxide liberated during the alcoholic fermentation; further by the expansion of air and the volatilisation of alcohol, water, and fatty acids formed by the bacteria. PETERS found four different budding fungi in leaven, and one of these has been identified with *Saccharomyces minor* (ENGEL). The second is of about the same size as *Saccharomyces minor*; the cells are egg-shaped, and in nutrient liquids develop to moderately large colonies with many branches; it yields spores abundantly. In addition to the above, a species of *Mycoderma* and a species related to *Saccharomyces cerevisiæ* were also found. PETERS describes several species of bacteria occurring in leaven, but none of them exhibits all the properties of LAURENT'S *Bacillus panificans*; these properties appear, indeed, to require distribution among various bacteria. LAURENT was probably, therefore, dealing with impure cultivations. These bacteria give no alcoholic fermentation, and no appreciable evolution of gas in sterilised dough.

LEHMANN has recently found, during his researches on leaven, that a single species of bacterium predominates, which he calls *Bacillus levans*. This bacterium forms lactic and acetic acids in sugar-broth, while carbon dioxide and hydrogen are disengaged; it is able to ferment sterilised flour. In certain ways this bacterium appears analogous to *Bacillus coli communis*. LEHMANN assumes that the yeast present in leaven is also active in the process.

The above experiments constitute a good preliminary to the decisive experiments on the cause and action of the rising of dough.

The diseases of black bread which have been investigated by UFFELMANN, KRETSCHMER, and NIEMITOWICZ, (*e.g.*, vigorous growth of mould, sliminess caused by an exuberant growth of bacteria), may no doubt be partly attributed to impure leaven, in which the most varied organisms will thrive.

SACCHAROMYCES ANOMALUS (HANSEN). (Figs. 42 and 68.)

This very curious species was found by HANSEN in an impure brewery yeast from Bavaria. It gives a rapid and vigorous fermentation in wort, and even at the beginning of the fermentation develops a dull grey film. During fermentation the liquid acquires an ethereal, fruity odour.

The cells grown in wort are small, oval, or sometimes sausage-shaped, and in their microscopic appearance they resemble the *Torula* species. When the development has gone on for some time many cells both in the sediment and in the film are found to contain spores.

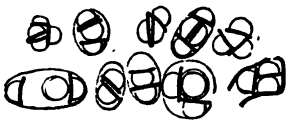

FIG. 68.—Saccharomyces anomalus in sporulation. Some spores are free, others inclosed in the mother-cells. On the right-hand side three spores are surrounded by the burst wall of the mother-cell (after HANSEN).

Spores are developed on various substrata, both liquid and solid, even under conditions where abundant nutriment is present.

The *form of the spores* is highly characteristic (Fig. 68); it resembles a hemisphere with a projecting rim round the base. On germination the spores swell and develop buds (see Fig. 42).

The maximum temperature for spore-formation is 32-32½° C., the minimum temperature 3-6° C., the optimum lying between 28 and 31° C. (17½-19 hours). (NIELSEN.)

After HANSEN had drawn attention to this curious *Saccharomyces* species, it was observed, together with other allied species, by HOLM, LINDNER, and WILL, who also found it in impure brewery yeast. Yeasts yielding hat-shaped spores appear in fact to be by no means uncommon.

In English high-fermentation beers, which were "fretty," the author observed a species belonging to this group, which was multiplying very freely, so that all other yeast-cells had

been repressed. It appeared distinctly as a disease-yeast causing turbidity in beer.

As previously mentioned, the spores of this fungus resemble those of *Endomyces decipiens*, and a relationship possibly exists between the two. As yet, however, no proof has been forthcoming in support of this assumption.

SACCHAROMYCES CONGLOMERATUS (REESS).

This species is described by REESS as follows:—"Round budding cells, of 5 to 6 μ diameter, united in clusters, which are formed from two old cells which, before budding in the direction of their common longitudinal axis, usually throw out simultaneously several buds as branches. The asci very frequently remain united in pairs, or each united to a vegetative cell. Spores 2 to 4, which on germination again give rise to clusters. Occurs on decaying grapes and in wine-yeast at the commencement of fermentation. Fermentative action doubtful."

In HANSEN'S cultures of film-formations of the *Saccharomycetes*, colonies having this appearance were found in old films of the six species first investigated. But as HANSEN never found a definite species among his cultivations which could be identified with REESS'S *Saccharomyces conglomeratus*, he is inclined to assume that the cell-colonies of the different *Saccharomycetes*, just described, are identical with this species.

In conclusion we must allude to a truly parasitical organism discovered by REMACK and ROBIN, and subsequently more closely examined by BUSCALIONI, viz.:

SACCHAROMYCES GUTTULATUS (BUSCAL.) (CRYPTOCOCCUS GUTTUL, ROB.),

which lives and multiplies in the intestinal canal of various mammals, birds, and reptiles. The growth consists of very large, oblong, or ellipsoidal cells, dark in colour and about 20 μ in length. Spore-formation was observed in the excrements of rabbits. The spores are oblong, about the same

shape as the mother-cells, and surrounded by a membrane with a distinctly double contour; they number from 1 to 4 in each cell, generally, however, only 2; they rather quickly detach themselves, and the membrane of the mother-cell then appears to be dissolved. The germination of the spores appears to take place only in the stomach of animals.[1]

A yeast species or variety is called a "**culture yeast**" if used industrially after a methodical selection made with special considerations in view. Many culture-yeasts exhibit microscopical differences from the "wild" yeasts occurring in the same branch of industry, as pointed out with regard to the construction of the spores.

The different races or species of yeast may be divided into two groups, according to the kind of fermentation to which they give rise, viz, *bottom-yeasts* and *top-yeasts*. In spite of many assertions to the contrary, it has not hitherto proved possible to bring about an actual conversion of top-yeast into bottom-yeast, or *vice versa*. The investigations of HANSEN and KUEHLE show that it is certainly possible for a bottom-fermentation yeast to produce transitory top-fermentation phenomena; these, however, quickly disappear with the pro-

[1] Concerning the behaviour of yeasts towards the *human and animal economy*, work has recently been carried out with pure cultures. SIMANOWSKY had pointed out that yeast in turbid beers may interfere with gastric and pancreatic digestion, and that such beers cause catarrh in the intestinal canal. NEUMEYER found that the yeasts examined by him were very resistant to the digestive juices, and could pass through the human and animal intestinal canal without being killed. If introduced into the intestinal canal without fermentable substances, neither culture-yeast nor wild yeast exercised any action; but when introduced along with sugar-solutions, in which they were able to produce a vigorous fermentation, they all of them caused stomachic and intestinal catarrh. This effect, according to NEUMEYER, is due to the fact that yeast, during its development at the high temperature of the body, secretes abnormal fermentation products which exert an injurious action upon the mucous coating of the intestinal canal. Recent experiments by FERMI and L. RABINOWITSCH have shown that yeasts exist which develop and multiply freely in the tissues on subcutaneous inoculation, and are capable of causing diseases which often end fatally. These species botanically should be classed with the so-called wild yeasts. Certain Monilia species have likewise proved to possess pathogenic properties.

gressive development of the yeast. The earlier statements, that by continued cultivation of, *e.g.*, bottom-yeast at a high temperature, it could be converted into top-yeast, can only be explained on the assumption that the bottom-yeast employed was impure and contained an admixture of top-yeast,

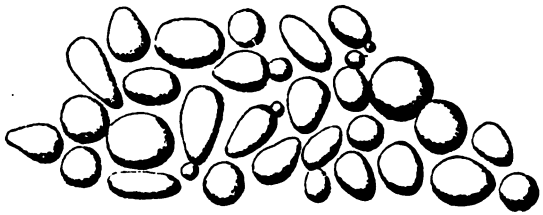

FIG. 69.—Carlsberg bottom-yeast, No. 1 (after HANSEN).

which, at the high temperature, gradually developed at the expense of the bottom-yeast, until it finally preponderated in the mixture.[1]

As examples of two different bottom-fermentation species

FIG. 70.—Carlsberg bottom-yeast, No. 2. Some cells with spores (after HANSEN).

of yeast, the *Carlsberg bottom-yeasts* "No. 1" (Fig. 69) and "No. 2" (Fig. 70), employed in the *Old Carlsberg* brewery at Copenhagen, may be more minutely described.

[1] LOISEAU and BAU have established an essential chemical difference between the *bottom*- and *top*-fermentation yeasts used in breweries. The former ferments melibiose and melitriose completely; the latter does not ferment melibiose, and decomposes melitriose into fructuose and melibiose, the former undergoing fermentation. According to E. FISCHER and these authors, melibiose is split up by an enzyme contained in bottom-fermentation yeast before being fermented. No such enzyme decomposing melibiose could be detected in the top-yeasts examined by FISCHER.

Distinct differences are noticeable even in an ordinary microscopic examination. The cells of species No. 1 (Fig. 69) are mostly somewhat elongated, but there are also smaller, characteristic, pointed cells. When the yeast taken from the fermenting-vessel is washed with water and placed for a short time under ice, the contents of all the cells quickly assume a granular appearance, and if the yeast is kept in this way for several days the number of dead cells will very rapidly increase. The cells of species No. 2 are, under normal conditions, roundish oval, some being almost spherical. Here and there giant-cells occur (left-hand side of figure). In a yeast-mass washed with water the cell-contents long remain clear, and only slightly granular, and if the yeast be kept for a long time in this way very few dead cells will be found.

The gelatine cultures of both species form colonies, having the ordinary appearance of the *Saccharomycetes.*

On gypsum blocks the species No. 2 develops spores much more quickly and abundantly than No. 1 species.

The fermentation phenomena also differ. No. 2 gives thick, high foam and a dense, firm layer on the surface; No. 1 gives a low foam, the liquor being often exposed in places. No. 2 clarifies comparatively quickly; No. 1 clarifies slowly. No. 2 forms a firm layer at the bottom of the fermenting-vessel, while No. 1 gives a fluid sediment. In the primary and secondary fermentations No. 2 gives a more feeble fermentation than No. 1.

The finished beer obtained in the same brewery with the two yeasts shows marked differences. With regard to taste, the beer obtained with No. 2 yeast is preferred by most, but this is a matter of opinion; at all events the taste is different in the two cases. Finally the two species give very different results as to the stability of the beers with regard to yeast turbidity. The beer prepared with No. 1 yeast is decidedly more stable in this respect than that prepared with No. 2 yeast. Consequently No. 1 is especially suited for lager and export beers, and No. 2 for running beers. These characteristics have been found to remain unchanged for years. (Compare BORGMANN's work on the chemical action of the two species.)

From this description of the microscopic relationships of these two types of bottom-yeast it must on no account be assumed that we are able, by means of a microscopic examination of an unknown species of yeast, to determine whether it will give a high or low attenuation, or whether it will clarify slowly or quickly, etc., etc. HANSEN'S and the author's investigations have, on the contrary, proved that *it is impossible to establish any general rule by this means,* for species which give a high attenuation may have the same microscopic appearance as species which give a low attenuation. It will only be possible to form an opinion of this character when our knowledge of the structure of the plasma is much more advanced. Statements to the contrary which have hitherto appeared in the literature of our subject are purely erroneous.

The comparison carried out by WILL of four bottom-fermentation yeasts may be instanced as a fine example of the application of HANSEN'S biological methods for the distinction of yeast-species. This comparison furnishes additional evidence of the fact that there exist distinguishable species within the limits of this group, as well as among those which have not yet found application in the services of industry. In his characterisation, WILL starts from the preliminary classification of brewery yeasts suggested by the writer in the year 1886, classing type 93 and 2 with the *strongly fermenting,* 7 with the *feebly fermenting* types, whilst type 6 is a yeast of *middle fermentation.* The four yeasts may be described as follows:—

Type 2 consists of large roundish or oval cells. Colonies on gelatine, globular or lenticular. Spores numerous and readily formed. Spore-formation at 11-31° C.; optimum, 25-26° C. Film-formation at 7-31° C.; very slow.

In *type* 6, oval cells predominate; but this species has a marked tendency to form sausage-shaped cells. Colonies on gelatine, globular or lenticular. Spores readily formed. Spore-formation at 11-31° C.; optimum, 28° C. Film-formation at 7-30° C.; slower than in type 7.

The cells of *type* 93 are typically oval, passing, however, generally into a roundish shape. Colonies in gelatine, globular or lenticular. Spores readily formed. Spore-formation at

10-30° C.; optimum, 28° C. Film-formation at 4-31° C.; very slow and weak. "Resting" cells very abundant in the film.

Type 7 possesses oval cells, often approximating to a spherical shape; "gigantic cells" regularly occur, and rich formation of chains of small oval cells at the end of fermentation. The young colonies on gelatine are irregular, sinuous and fringed. This variety does not readily develop spores. Spore-formation at 13-30° C.; optimum, 25-26° C. Film-formation at 4-28° C.; begins sooner than in the other varieties. Few "resting cells" in the film.

A series of ellipsoidal *wine-yeast* species were characterised morphologically and biologically by ADERHOLD. KAYSER also described a considerable number of *wine-yeasts* from different countries, as well as the various species occurring in *cider*-fermentation.

By way of examples illustrative of the considerable differences which may occur among *high-fermentation culture-yeasts*, may be mentioned the following nine species of *English high-fermentation yeast*, which were prepared in a state of absolute purity in the author's laboratory, and described by him in conjunction with J. CH. HOLM:—

The two morphological and biological stages—the alcoholic fermentation and the film- or mould-stage—were pictured and described from growths developed in the following manner:

The *alcoholic fermentation stage* from growths which had first been kept for a considerable length of time in a 10 per cent. saccharose solution, then cultivated during several generations in brewery-wort, and finally developed in the same liquid in Pasteur flasks for 24 hours at a temperature of 25° C.

The *development* of the *films* and their *appearance* to the *naked eye* were studied by means of growths in brewery-wort in Erlenmayer flasks at the room temperature (about 20° C.).

The *cell-forms* of the *films* were observed by means of growths in Pasteur flasks at 20° C. after five months' culture.

The condition of the deposited yeast was estimated by growths in Pasteur flasks at the room-temperature.

The fermentation experiments were conducted at room-

temperature with sterilised hopped wort in high cylindrical vessels, covered up with several layers of sterilised filter-paper; after completion of primary fermentation the liquids were introduced into sterilised bottles, which were kept at a lower temperature. The amounts of alcohol produced (1) when the primary fermentation was interrupted, (2) during the first two weeks of the after-fermentation, and (3) during the next two weeks, were determined. The principal fermentation was broken off as soon as it was judged by the appearance of the cells that the first vigorous development had come to a stop. In making this comparison we did not attempt to solve the question as to the *absolute* amount of alcohol which these species are able to produce during primary and secondary fermentation, our sole object being to institute a comparison between the species.

The flavour of the fermented liquid was determined after the beer had gone through a secondary fermentation at a lower temperature in bottles, which were first closed with cotton-wool plugs and afterwards with ground-glass stoppers.

1. *Fig.* 71, 1 *and Fig.* 73, 1.

The cells during fermentation are comparatively small, oval, linked in chains; among them there occur big round and grotesque forms.

The yeast lies rather loose in the flask; if shaken it does not distribute itself equally in the wort, but separates into clots.

Film-formation: After a lapse of 31-32 days a very thin film covers almost the whole surface of the liquid.

The cells of the film are of about the same size as those seen during the primary fermentation; some cells very much lengthened.

The spores, if developed at a low temperature, are small, full of vacuoles, and slightly granulated; as a rule only one or two in each cell.

At 11-12° C. a few spores make their appearance on the seventh day; at 25° C. abundant development of spores in 40 hours.

When the principal fermentation was broken off, the liquid

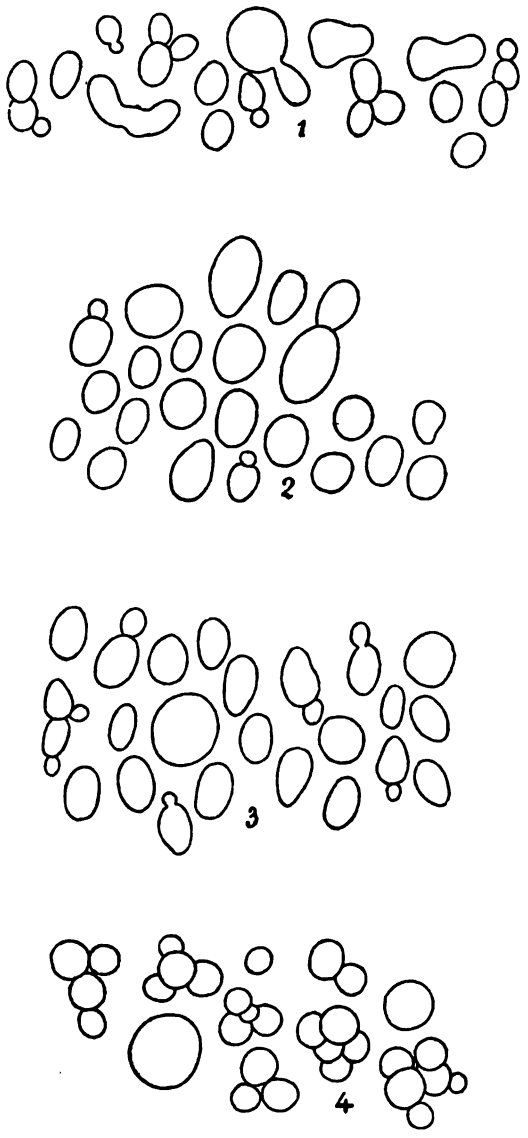

FIG. 71, 1-4.—Young vegetations of English top-yeasts. (HOLM.)

contained 2·49 per cent. (by vol.) of alcohol; during the two following periods (see above) 0·31 and 0·57 per cent. (by vol.) were produced.

Production of acid, after expulsion of CO_2, corresponding to 5·0 c.c. of decinormal caustic soda solution.

The fermented liquid has an agreeable smell and a fine aromatic taste.

2. *Fig.* 71, 2 *and Fig.* 73, 2.

During fermentation most cells are free, medium-sized, round and oval; among themthere occur round and oval gigantic cells.

The yeast lies loose in flask; if shaken slightly, it is distributed like a cloud throughout the liquid.

Film-fermentation: After 31-32 days, a few large patches.

The cells of the film are smaller than those seen during primary fermentation; ellipsoidal and slightly lengthened.

The spores, if developed at a low temperature, are big; formation of partition walls occurs readily.

At 11-12° C. very few spores occur on the seventh day; at 25° C. rather abundant spore-formation in 40 hours.

When the principal fermentation was broken off, the liquid contained 2·30 per cent. (by vol.) of alcohol; in the two following periods 1·00 and 0·46 per cent. (by vol.) were formed.

Acid-production: 6·0.

Disagreeable smell and taste.

3. *Fig.* 71, 3 *and Fig.* 73, 3.

During fermentation the growth shows free cells and small chain-formations of oval forms; a few globular gigantic cells.

The yeast lies very compact in flask; only on violent shaking it partially rises in the liquid.

Film-formation: In 31-32 days the growth forms a very thin film, which does not cover the entire surface of the liquid.

Some of the cells of the film have the same size and shape as those seen during primary fermentation; others are slightly lengthened.

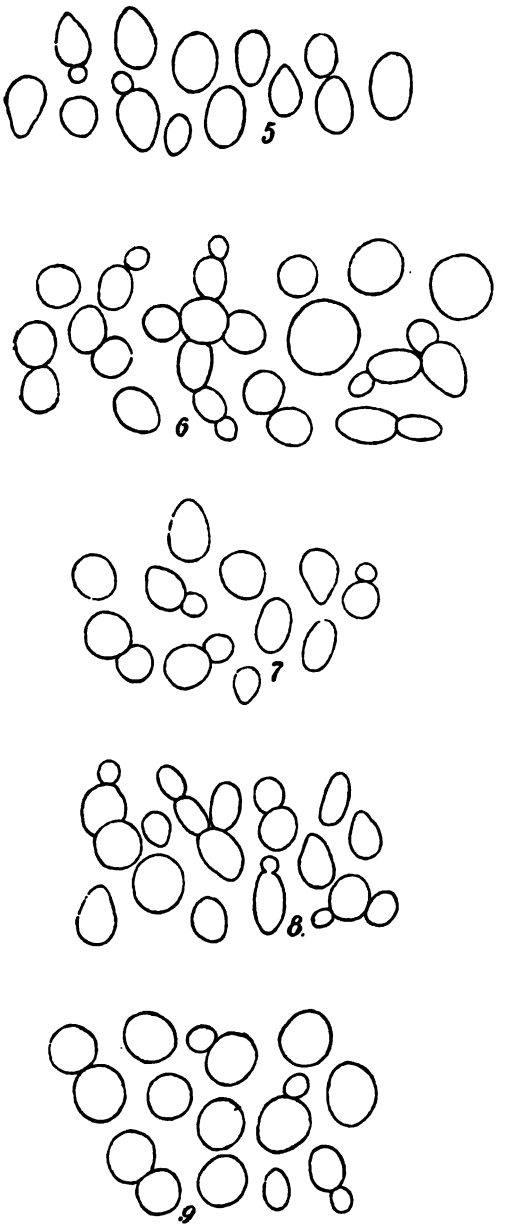

FIG. 72, 5-9.—Young vegetations of English top-yeasts. (HOLM.)

The spores, if developed at a low temperature, are of very different sizes, comparatively feebly refractive, without distinct vacuoles. Partition-wall formations occur. At 11-12° C., in 7 days, only rudiments of spores appear; at 25° C., in 40 hours, spores are very freely formed.

When the principal fermentation was broken off, the liquid contained 2·26 per cent. (by vol.) of alcohol; during the following two periods 0·79 and 0·00 per cent. (by vol.) were formed.

Acid-production: 5·5.

Disagreeable smell and taste.

4. *Fig.* 71, 4 *and Fig.* 73, 4.

During fermentation there occur colonies consisting of many small globular cells; among these cells of a gigantic size occur.

The yeast lies loose in flask; if slightly shaken, it is distributed like a cloud throughout the whole liquid.

Film-formation: After 31-32 days, slight beginnings.

The cells of the ring-growth occur in colonies, which sometimes contain upwards of a hundred cells, all descended from one cell; only the youngest growths are long and very narrow.

The spores, if developed at a low temperature, are small and vacuolized. At 11-12° C., even after a fortnight, no spore-formation; at 25° C., for 40 hours, a very scanty development of spores.

When the principal fermentation was broken off, the liquid contained 1·80 per cent. (by vol.) of alcohol; during the following two periods 1·00 and 0·82 per cent. (by vol.) were formed.

Acid-production: 5·5.

Disagreeable smell and taste.

5. *Fig.* 72, 5 *and Fig.* 74, 5.

During fermentation most cells are free, medium-sized, and egg-shaped.

The yeast lies rather loose in flask; if shaken, it is not distributed equally in the wort, but separates into clots.

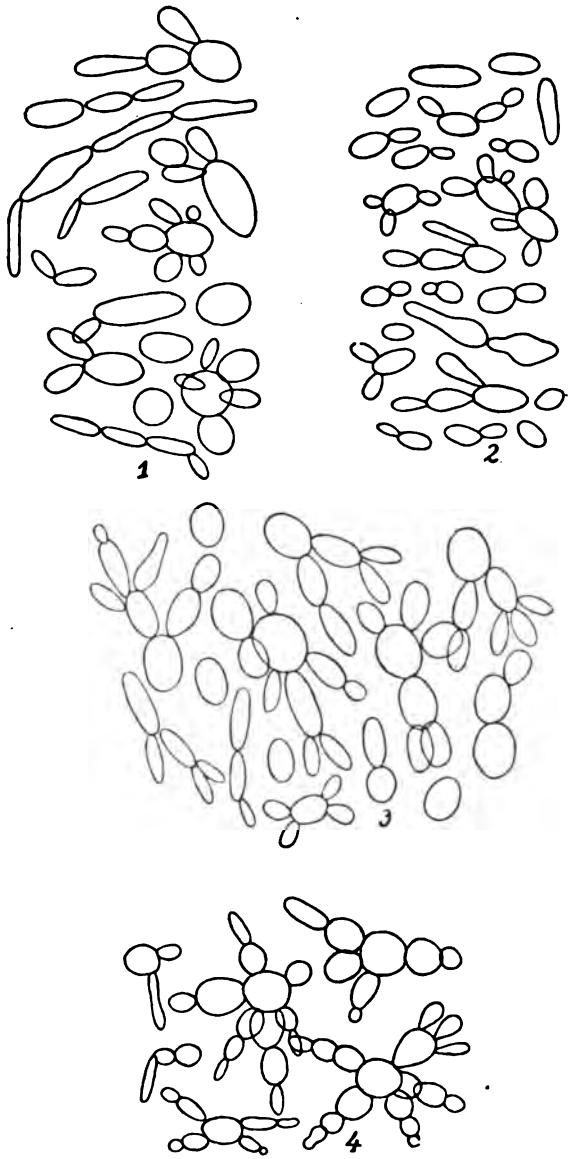

FIG. 78, 1-4.—Film-formations of English top-yeasts.

Film-formation: After 31-32 days a distinct film, which, however, does not cover the whole surface, and which subsequently develops slowly.

The cells of the film have a very different appearance from those seen in the fermentation-stage. Many of them are much lengthened, irregularly wound in tortuous curves, and some have developed a ramified mycelium.

The spores, if developed at a low temperature, are small, coherent, granulated. At 11-12° C. no spores appear even after a fortnight; at 25° C., after 40 hours, a very scanty spore-formation takes place.

When the principal fermentation was broken off, the liquid contained 2·49 per cent. (by vol.) of alcohol; in the following two periods 0·86 and 0·12 per cent. (by vol.) were formed.

Acid-production: 5·2.

Agreeable smell and fine aromatic taste.

6. *Fig.* 72, 6 *and Fig.* 74, 6.

The cells are round, oval, and elongated during fermentation, all forms occurring in chain-formations; single round gigantic cells occur.

The yeast lies rather compact in flask; it requires strong shaking to distribute the cells equally throughout the liquid.

Film-formation: After 26 days the growth formed a ring of yeast-cells on the surface, against the wall of the flask; only slight indications of film-formation. After 31-32 days the film had not developed further.

The cells of the ring-growth do not distinguish themselves essentially from those occurring during the alcoholic fermentation.

The spores, if developed at a low temperature, are comparatively small, granulated, with no distinct vacuoles. At 11-12° C., for 7 days, only very few spores are formed; at 25° C., for 40 hours, a scanty spore-formation takes place.

When the principal fermentation was broken off, the liquid contained 1·85 per cent. (by vol.) of alcohol; during the following two periods 0·65 and 0·20 per cent. (by vol.) were formed.

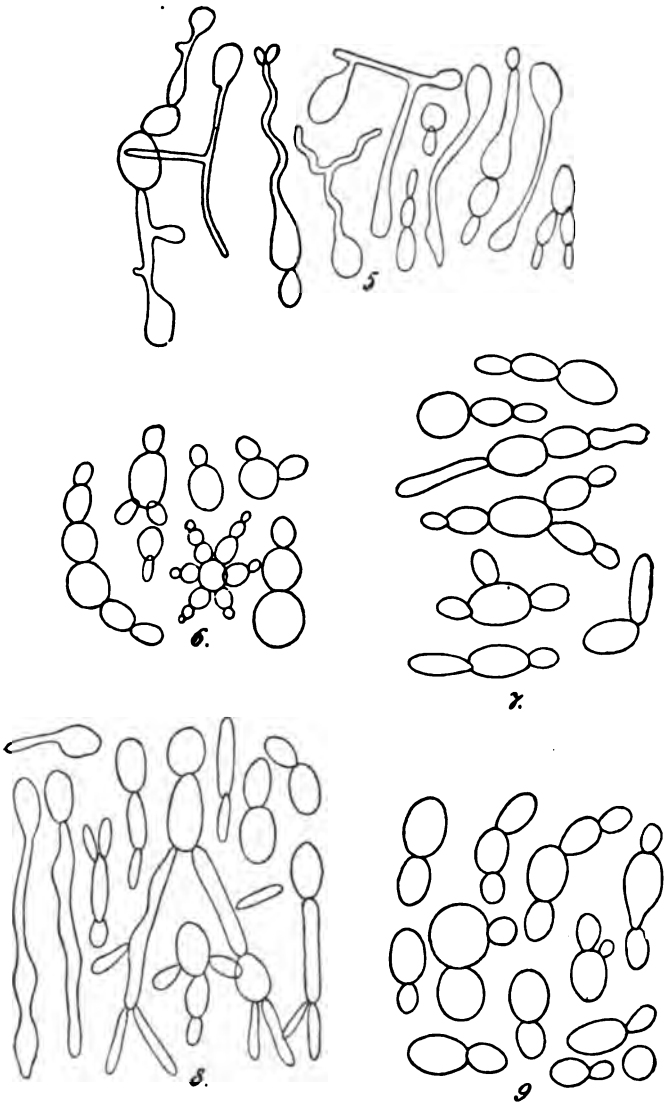

FIG 74, 5-9.—Film-formations of English top-yeasts.

Acid-production: 6·0.

Agreeable smell and slightly aromatic taste.

7. *Fig.* 72, 7 *and Fig.* 74, 7.

During fermentation round and oval cells, some free, others linked together in short chains.

The yeast lies rather compact in flask; violent shaking is required to distribute the cells equally throughout the liquid.

Film-formation: After 26 days a thin, almost continuous film appears, which in the course of the next 5-6 days grows to form a conspicuous covering extending over the whole surface of the liquid.

The cells of the film have in the main the same shape as those seen during fermentation; only the youngest generations are lengthened and narrow.

The spores, if developed at a low temperature, are medium sized, with no distinct vacuoles. At 11-12° C., after 9 days, fully developed spores appear; at 25° C., for 40 hours, spores are formed freely.

When the principal fermentation was broken off, the liquid contained 2·40 per cent. (by vol.) of alcohol; during the following two periods 0·95 and 0·00 per cent. (by vol.) were formed.

Acid-production: 6·5.

Agreeable smell and slightly aromatic taste.

8. *Fig.* 72, 8 *and Fig.* 74, 8.

During fermentation round, oval, and elongated cells, both free and linked together.

The yeast lies rather compact in flask; on violent shaking the cells are distributed equally throughout the liquid.

Film-formation: After 31-32 days very slight isolated indications of a film on the surface, and a ring of yeast-cells on the glass, round the edge of the liquid.

The cells of the film have assumed quite different shapes from those of the fermentation-stage; they are very much lengthened, mycelium-like and irregular.

The spores, if developed at a low temperature, are medium-sized, with no distinct vacuoles. At 11-12° C. spores are formed pretty freely on the ninth day; at 25° C., for 40 hours, they are formed freely.

When the principal fermentation was broken off, the liquid contained 2·77 per cent. (by vol.) of alcohol; during the following two periods 0·98 and 0·00 per cent. (by vol.) were formed.

Acid-production: 6·5.

Odour good, but bitter, adherent taste.

9. *Fig.* 72, 9 *and Fig.* 74, 9.

During fermentation a very uniform growth of big, round and oval cells.

The yeast lies rather loose in flask; on violent shaking the cells are distributed equally throughout the liquid.

Film-formation: In 31-32 days very slight, isolated indications of film-formation on the surface, and a slender ring of yeast-cells on the glass, round the edge of the liquid.

The cells of the film differ but little from those of the fermentation.

The spores, if developed at a low temperature, are medium in size and granulated. At 11-12° C. spore-formation sets in on the ninth day; at 25° C., for 40 hours, a not very abundant spore-formation takes place, accompanied by a considerable formation of net-work.

When the principal fermentation was broken off, the liquid contained 2·96 per cent. (by vol.) of alcohol; during the following two periods 1·19 and 0·00 per cent. (by vol.) were formed.

Acid-production: 7·0.

Odour good, markedly vinous taste.

A preliminary *grouping for practical purposes* of the different species or races of *bottom-* and *top-fermentation beer-yeasts* which have been prepared in pure culture by HANSEN'S method in the author's laboratory, is as follows:

A.—Bottom-fermentation Species.

1. Species which clarify very quickly and give a feeble fermentation; the beer holds a strong head. The beer, if kept long, is liable to yeast-turbidity. Such yeasts are only suitable for draught-beer.
2. Species which clarify fairly quickly and do not give a vigorous fermentation; the beer holds a strong head; high foam: the yeast settles to a firm layer in the fermenting vessel. The beer is not particularly stable as regards yeast-turbidity. These yeasts are suitable for draught-beer and partly for lager-beer.
3. Species which clarify slowly and attenuate more strongly. The beer is very stable against yeast-turbidity. These yeasts are suitable for lager beer, and especially for export beers.

B.—Top-fermentation Species.

1. Species which attenuate slightly and clarify quickly. The beer has a sweet taste.
2. Species which attenuate strongly and clarify quickly. Taste of beer more pronounced.
3. Species which attenuate strongly and often clarify slowly. The beer is stable against yeast-turbidity.

By far the greater number of high-fermentation yeasts examined in this respect *are able to carry through a secondary fermentation.* In classes 2, and especially 3, this secondary fermentation is very vigorous and long continued.

As a very significant result of practical experience, and one which shows how pronounced are the characters of many species of cultivated yeast, it may be mentioned that, speaking generally, *the above grouping holds good, even under the different practical conditions obtaining in widely separated countries.* For instance, the Carlsberg yeast No. 1 gives everywhere a beer which is very stable as regards yeast-turbidity; other species, which clarify more rapidly, have been found to retain this property everywhere under normal brewery-conditions. This

is the chief result of the writer's experience, extending over many years; it has rendered it possible to compare the very varied circumstances obtaining in the different beer-producing countries.

An example of the permanence of the specific properties under very different external conditions has also been given by IRMISCH in a comparative examination of two bottom-yeasts. One of the species gave a low attenuation and multiplied to a very small extent in the wort, whilst the other, on the contrary, gave a high attenuation and possessed the power of multiplication in a high degree; the course of the fermentation in the two cases also showed marked differences. These differences still existed on varying the concentration of the wort or the quantity of the yeast, at very different temperatures, and in every kind of medium, thus:—when cultures were employed which have been grown in wort containing diastase; under various conditions of aëration with ordinary wort; with a specially prepared wort very poor in maltose; in the presence of grains during fermentation; and in solutions of cane-sugar. Even in fermentations which had been carried on for six months, an examination of the product showed that the typical differences of the two species had not disappeared.

Certain high-fermentation species are employed in *distilleries* and in *yeast factories*. Since 1887 a number of distillery yeasts, yeasts for the fermentation of molasses and the manufacture of pressed yeast, have been prepared in the author's laboratory in pure culture. They exhibit marked constant differences in their sedimentary forms and in ascospore-formation. The species which have been introduced into practice also differed in this respect. DELBRUECK, P. LINDNER, and STENGLEIN have since had the same experience.

According to the writer's experience, these yeast-species also exhibit characteristic differences among themselves. Indeed, such differences may be observed in the *general appearance of the fermentation*, some species showing decided *top-fermentation* phenomena, while others in parallel experiments behave as *bottom-fermentation* yeasts. This diversity also appears in the *power of multiplying* of the cells and in the *yield of alcohol*

under parallel conditions; and lastly, a well-marked difference between the species is noticeable in their behaviour towards the wide range of nutrient liquids used in these branches of industry (barley- and rye-mash, maize- and potato-mash, molasses, etc.). Hence, it is essential that types suited to the existing circumstances should always be selected.

The condition of the *product of fermentation* exhibits considerable differences, as has further been shown by the extensive investigations of RAYMAN and KRUIS.

Morphologically, the author finds that a curious difference appears between the species used in practice, viz.: that they are naturally grouped in two classes, of which one ("A") has very *short chains of cells*, the buds being very soon detached from the mother-cells; whilst the other ("B") exhibits *large chains consisting of many cells*. This typical character has remained unaltered in species which were isolated in pure cultures ten years ago and used in practice. There does not exist any direct connection between this morphological phenomenon and the nature of the fermentation, whether top or bottom.

BÊLOHOUBEK, SCHUMACHER, and WIESNER, have also carried out microscopical and chemical investigations into these yeasts, and BÊLOHOUBEK'S "Studien über Presshefe" (Prague, 1876) contains specially accurate descriptions of the appearance under the microscope of pressed yeast in the different stages of its development, and observations on the microscopic indications of the quality of manufactured yeast, so far as can be judged from the cell-contents. The decomposing yeast-cells show a change in the colour and consistence of their plasma; this gradually turns darker and liquid, the vacuoles grow larger, and the sharp outlines between the vacuoles and the plasma gradually disappear, the plasma shrinks from the cell-wall and finally collects in irregular masses in the cell-fluid; these also disappear at last, and finally the cell-wall is dissolved. According to these authors, cells also occur in pressed-yeast, which suddenly develop a number of small vacuoles; these "abnormal vacuolar" cells quickly perish.

OTHER BUDDING-FUNGI.

(*Torula, Saccharomyces apiculatus, Mycoderma cerevisiæ and vini.*)

In the following pages we give a review of some other fungi, which are of more or less importance in the fermentation industries, and which resemble the *Saccharomycetes* so far as they multiply by budding; these species develop a *mycelium* only exceptionally. On the other hand they are all distinguished from the *Saccharomycetes* by the absence of the power of forming endogenous spores which characterises the latter.

The forms examined by HANSEN, which produce a mycelium, should strictly be classed with the mould-fungi. Since, however, their position amongst the moulds has not yet been systematically determined, these species may, for practical reasons, be described in this place.

TORULA (HANSEN).

These yeast-like forms were first characterised by HANSEN. They are widely distributed and therefore not infrequently occur in physiological analyses connected with fermentation. They occur in both spherical and more or less elongated forms, and are distinguished from the genus *Saccharomyces*, as first pointed out by HANSEN, by their inability to form endogenous spores. In most cases they multiply only by budding, in some few cases also by the formation of mycelium.

According to the author's researches, certain *Torula* forms may act as *disease-yeast*, for they multiply freely and give rise to a kind of turbidity in *weakly fermented high-fermentation beers*, when these are bottled; the character of this turbidity, however, is somewhat different from that caused in low-fermentation beer by the wild *Saccharomycetes*.

In sugar-works, the writer finds *Torula* forms occurring extensively, frequently in large quantities, even in the finished produce. Among the species examined many possessed an

inverting enzyme. It is not improbable that these growths assist in the progressive formation of invert-sugar which frequently takes place during the warehousing of cane-sugar.

HANSEN has observed many different species, and has described the following in detail:—

The *first* occurs in wort, the cells being either single or in small clusters. Some cells have a large vacuole in the middle, and this sometimes contains a small strongly-refractive particle. The size of the cells varies considerably (1·5 to 4·5 μ). This species does not secrete invertase, and causes a scarcely perceptible alcoholic fermentation in beer-wort.

The *second species* has, under the same conditions, larger cells (3 to 8 μ) than the first; they resemble the foregoing,

FIG. 75.—Torula: sedimentary forms after one day's cultivation in beer-wort at 25° C. (After HANSEN.)

except that the contents of the cells grown in wort are often very granular.

The *third species* which, under the microscope, resembles the last, produces under the same conditions as much as 0·88 per cent. by volume of alcohol; it gives a distinct head with evolution of carbon dioxide, but it cannot invert cane-sugar.

The *fourth species* (2 to 6 μ) inverts cane-sugar and produces a little more than 1 per cent. by volume of alcohol in wort with considerable frothing; it does not, however, ferment maltose.

The *fifth species*, which in the form and size of its cells resembles the first, develops a uniform, dull grey film on wort and yeast-water at the ordinary room temperature, likewise on lager beer and even on liquids containing as much as 10 per cent. of alcohol. It inverts cane-sugar and forms a slight film on the solution. It does not, however, excite any appreciable alcoholic fermentation.

A *sixth species* (Fig. 75), which forms spherical and oval

cells, gives a distinct fermentation in beer-wort, yielding as much as 1·3 per cent. by volume of alcohol. It does not ferment maltose solutions. It inverts cane-sugar, and in 10 per cent. and 15 per cent. solutions of this sugar in yeast-water, it yields respectively 5·1 and 6·2 per cent. (volume) of alcohol after fourteen days' cultivation at 25° C.; the last

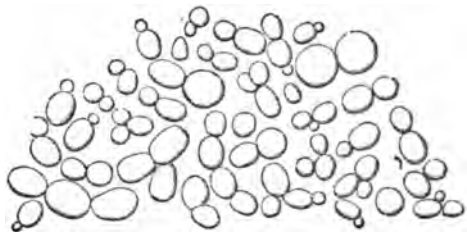

FIG. 76.—Torula: sedimentary forms after one day's growth in beer-wort at 25° C. (After HANSEN.)

growth yielded 7 per cent. (volume) of alcohol in two months. Dextrose solutions of the same concentration and under similar conditions gave 6·6 and 8·5 per cent. of alcohol by volume.

The *seventh species* (Figs. 76 and 77) was found in the soil under vines. The sedimentary cells are most frequently oval

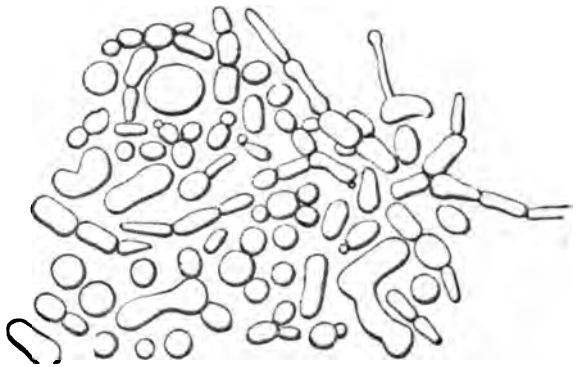

FIG 77.—Same species as Fig. 76. Film-formation on a wort-culture ten months old. (After HANSEN.)

and in part larger than those of the last species. Certain cells of the films are very irregular in form. This *Torula* produces only 1 per cent. (volume) of alcohol in wort, does not

ferment maltose, and neither ferments nor inverts cane-sugar In 10 per cent. and 15 per cent. solutions of dextrose in yeast-water it gives 4·6 and 4·5 per cent. by volume of alcohol after 15 days at 25° C., and 4·8 and 4·7 per cent. in 28 days. In two other flasks 4·8 and 5·3 per cent. of alcohol had been produced after long standing. HANSEN assumes that this species takes part in vinous fermentation, and considers it probable that species such as the sixth and seventh, which produce a vigorous fermentation in dextrose solutions, take part in the fermentation of grape-juice and other fruits. On the other hand they have probably little importance in breweries and distilleries, since they are unable to ferment maltose.

Another species of *Torula* (*Torula Novæ Carlsbergiæ*), the cells of which exhibit very different forms, has been described by GROENLUND. It imparts a disagreeable bitter taste to wort. According to SCHJERNING'S investigations it inverts cane-sugar, and induces alcoholic fermentation in solutions of cane-sugar, dextrose, and maltose. In ordinary brewery-wort it can produce about 4·7 per cent. (volume) of alcohol.

Torula species which contain no invertase, yield only about 1 per cent. (volume) of alcohol, and do not ferment maltose, are found widely distributed in nature. Those which have been examined ferment solutions of dextrose.

Related to the above are the *red budding-fungi* (the "*pink yeast*" of medicinal bacteriology) universally distributed in atmospheric dust; several species of these are known. KRAMER, for instance, found in must a top-fermentation torula-yeast which produces a red colouring-matter soluble in water. It ferments dextrose, and, in a 10 per cent. solution, it yields 4·5 per cent. by volume of alcohol; it inverts cane-sugar, directly ferments maltose, but has no action on lactose.

These different species cannot be distinguished by the microscope alone either from each other or from the round cells of the *Saccharomycetes*, and, as seen above, there are species with pronounced fermentative activity.

HANSEN assumes, with some degree of probability, that they are derived from the higher fungi, and in his cultivation experiments he has observed the development of a *mycelium* in a few cases.

TORULA-YEASTS FERMENTING MILK-SUGAR.

DUCLAUX found a yeast-fungus in milk which induces *alcoholic fermentation in a solution of lactose.* This fungus appears to be most closely related to the *Torula* species. The cells are 1·5 to 2·5 μ in diameter, and almost spherical. According to DUCLAUX'S experiments, this yeast is more aërobic than the ordinary alcoholic yeasts. Even with strong aëration of the liquid, the whole of the milk-sugar is used up in the alcoholic fermentation. In a 5 per cent. solution of milk-sugar 2·5 per cent. of alcohol was formed in eleven days at 25° C. The most favourable temperature for the fermentation of a neutral solution is 25° to 32° C., whilst at 37° to 40° C. the fermentation ceases. Small quantities of acid have a retarding influence on the fermentative activity of this yeast.

ADAMETZ likewise describes a budding-fungus which ferments *milk-sugar* (*"Saccharomyces lactis"*). Since this fungus does not yield endogenous spores by HANSEN'S method, it is classed in the group of *non-Saccharomycetes.* The cells are of about the same size as those of *Saccharomyces cerevisiæ*, and are spherical and elliptical. The colonies grown on peptone-gelatine are round, with slightly jagged borders, and are of a dark brown colour. A puncture-cultivation in wort-gelatine yields a dull, flat mass on the surface and a vigorous growth in the punctured channel, and from this numerous offshoots penetrate into the gelatine. In sterilised milk this fungus induces fermentation phenomena within 24 hours at 50° C., in 48 hours at 38° C., and in about four days at 25° C. In this fermentation the milk-sugar is alone decomposed.

Both of the species mentioned above have been more closely investigated by KAYSER, together with a *new species* which likewise ferments lactose and belongs to the *non-Saccharomycetes.* All three yield colonies on gelatine, which are more widely spread than those of beer- and wine-yeasts; in the middle of the colonies there is a thick portion, while the border resembles mycelium. In milk and in neutral liquids, when sufficiently aërated, they induce an appreciable fermentation at 25° to 30° C. The milk does not coagulate or

become viscous during the alcoholic fermentation. All three species ferment lactose, galactose, cane-sugar, glucose, invert-sugar, and finally maltose, but the last only with great difficulty. In the fermentation of milk-sugar with these yeasts, the resulting liquids are as rich in alcohol as the strongest beers. KAYSER remarks that it may, perhaps, be possible to make practical use of this observation and by means of these fungi convert the large quantities of whey, obtained in the manufacture of cheese, into an alcoholic liquor.

BEIJERINCK has described two yeasts which also ferment milk-sugar, and which must be provisionally regarded as *non-Saccharomycetes*; these are "*Saccharomyces Kephir*," which occurs in kephir-grains and consists of longish cells of varied shape, and forms slightly jagged colonies liquefying gelatine; and "*Saccharomyces Tyrocola*," which consists of small roundish cells, and forms snow-white colonies on gelatine. BEIJERINCK found that these two species secrete a particular invertive ferment (*lactase*) which inverts not only cane-sugar but also milk-sugar; it does not, however, invert maltose. It is stated that lactase may be prepared as follows:—A five per cent. solution of milk-sugar, containing nutrient salts and asparagine, is fermented with kephir-yeast; the product is filtered and the ferment is precipitated from the filtrate by the addition of alcohol. According to SCHUURMANS STEKHOVEN, however, the enzyme of BEIJERINCK'S kephir-yeast does not invert milk-sugar.

In Lombardy cheese a unilaterally budding top-fermentation yeast was discovered by BOCHICCIO, which is called *Lactomyces inflans caseigrana*. It causes blisters on the surface of hard cheese. The growth consists of round, ellipsoidal and oblong cells, and forms whitish colonies on gelatine, with smooth edges. No spore-formation was observed. The fungus coagulates sterilised milk, and partly liquefies the coagulum without any decided formation of acid. In lactose-broth it produces a vigorous fermentation at 25-40° C.; the best temperature for the development is about 30° C., the limit of existence at about 60° C. Whey infected with this species is converted into a foaming beverage of a somewhat agreeable taste.

SACCHAROMYCES APICULATUS (REESS). (Fig. 78.)

According to our present views, the name of this ferment is incorrect, for only those budding-fungi which yield endogenous

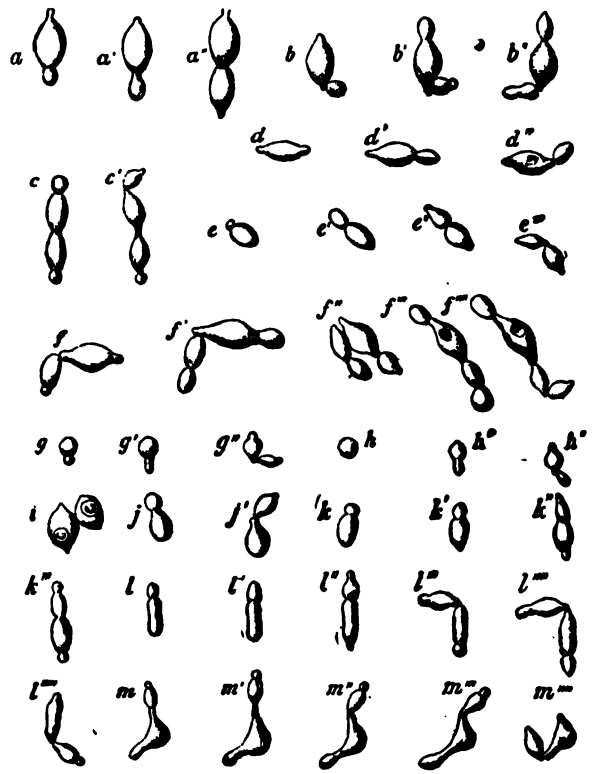

FIG. 78.—Saccharomyces apiculatus (after HANSEN). Budding cells: *a*—*a*″, a cell which in the course of 3½ hours developed a bud at its lower extremity; *b*—*b*″, a similar series, showing the development of a bud at the upper extremity of the mother cell, whilst a bud had been previously formed at the opposite end; *c* is a chain of cells, *c*′ is the same three-quarters of an hour later; the lowest bud had, like those above it, assumed the typical form of the species, but in the figure it is seen from the end, so that its longitudinal axis is at right angles to the plane of the paper; *d*—*d*″, development during 1½ hours; *e*—*e*‴, during 2½ hours; *f*—*f*⁗, during 3 hours; in *e*—*f*, it is seen that the oval cells first develop a bud and only subsequently assume the typical lemon-shape; *g*—*m*, abnormal cell and series of development.

spores are considered to belong to the *Saccharomycetes*, and the fungus in question does not possess this property. We will, however, provisionally retain the old generic name, as has been

done by HANSEN, until systematic classification has been further developed.

This ferment was the subject of one of the finest and most thorough biological investigations of our time, for HANSEN was enabled, after several years' work, to determine both its habitat in nature and its regular migrations at the different seasons of the year. The reason why this species was selected for the investigation was that, while other species occur in very varied and uncertain forms, making the study of their occurrence in different localities very difficult, this ferment can be recognised with certainty, for it always occurs in cultures with lemon-shaped cells; this is the typical form of the species.

Sacch. apiculatus occurs abundantly in wine-yeast, especially during the first stages of the fermentation, also in spontaneously-fermented Belgian beer; in nature it is found abundantly on ripe, sweat, succulent fruits.

If a little of such a growth is examined under the microscope in a drop of nutritive liquid, the development of the fungus can be followed. This is very characteristic (compare Fig. 78). It is seen that the buds formed from the typical lemon-shaped cells may be either lemon-shaped (*a, b, c, e, f*) or oval (*a—c*); it is also noticed that the oval cells must first form one or more buds before they are able to assume the lemon-shape (*e—f*), and finally, that the lemon-shape of a cell attained by budding (*k, k', k''*) may be lost again on the formation of a new bud (*k'''*). Under other conditions the cells may assume quite different forms, sausage-shaped, half-moon-shaped, bacteria-like, etc. (*g—m*). Now, does any rule govern this apparent confusion of forms? It has just been shown that the fungus can form two kinds of buds, and that the oval buds must develop one or more new buds before they can assume the typical form. The question then is: Under what conditions are those two kinds of buds developed? It was shown by means of culture experiments that the lemon-shaped buds are developed especially during the first stages of the culture, but are afterwards crowded out by the oval forms.

We shall now give a further description of the fungus from a physiological and biological standpoint.

Saccharomyces apiculatus is a bottom-fermentation yeast,

capable of exciting alcoholic fermentation in beer-wort; the fermentation in this liquid is, however, a feeble one, only 1 per cent. by volume of alcohol being produced, whilst *Saccharomyces cerevisiæ* (bottom-yeast) under the same conditions gives 6 per cent. This arises from the fact that *Sacch. apiculatus cannot ferment maltose.* HANSEN also found that it *does not secrete invertase.* On the other hand it excites a vigorous fermentation in 15 per cent. and 10 per cent. solutions of dextrose in yeast-water, and in one experiment as much as 3 per cent. by volume of alcohol was formed. After three months the liquid still contained sugar, whilst the amount of alcohol had not increased during the last one and a half months. The fungus was thus unable to complete the fermentation. In another of HANSEN'S experiments as much as 4·3 per cent. by volume of alcohol was produced.

It was found from experiments, in which a mixture of this fungus with *Saccharomyces cerevisiæ* was grown in beer-wort, that it was crowded out by the latter, being the weaker species, although it retarded the *Sacch. cerevisiæ* to no small degree.

In flasks with the same beer-wort, and at the same temperature, each containing one species, *Saccharomyces apiculatus* will multiply to a greater extent than *Saccharomyces cerevisiæ* in a given interval of time.

At the critical time of the year, the ferment, if present in the wort in considerable quantities, may exist for a length of time side by side with *Saccharomyces cerevisiæ*, and will no doubt retard its action a little; but when the beer is transferred to the lager cellar, the fungus remains inactive in the alcoholic liquid and frequently perishes.

According to WILL, the fungus frequently occurs in slight traces in low-fermentation beer-yeast; it may be caused to multiply more freely by treatment with tartaric acid, and is, thus, easily found by microscopical examination. He found differences in the pure cultures of apiculate-yeasts, for some develop a strong bouquet resembling an amyl-ester in wort, whilst others produce a peculiar mouldy smell.

MUELLER-THURGAU and WORTMANN regard the fungus as injurious to wine, for it not only directly prejudices the

quality of the wine and must, but also checks the fermentation and thus gives rise to disease.

The most interesting phases in the life of this ferment are the *conditions of its occurrence in nature*, which have also been explained by HANSEN. Microscopic investigations and culture-experiments have shown that in summer the ferment was *abundantly developed on sweet, succulent fruits* (cherries, gooseberries, strawberries, grapes, plums, etc.) *during their ripening.* On the contrary, it was only very exceptionally found on the same fruits so long as these were unripe. Since it is found in a vigorous condition of budding on these ripe fruits, but is never or only exceptionally found on other fruits, leaves, twigs, etc., it is perfectly clear that *Saccharomyces apiculatus* has its true habitat on ripe fruit. This was further proved by the fact that it always, without exception, occurs in the soil under the cherry trees, plum trees, vines, and other plants on the fruits of which it is found; whilst, on the other hand, it was only extremely seldom that it was found in the numerous samples of soil from other and most diverse localities. The ferment is carried into the earth at such places by the fallen fruit and by rain, and the question then arises whether it also winters there. The answer was obtained in two ways: partly by taking numerous samples of soil from these places during winter and spring—these, when introduced into flasks containing wort, gave a vigorous growth of our ferment in the great majority of cases—partly by introducing, with every precaution, cultures of *Saccharomyces apiculatus* into the soil and leaving them during the winter. In the spring and early summer the soil was again examined, and culture experiments proved that the ferment was still alive in all the samples. Thus, it was proved that the ferment *can* winter in the soil, and it had previously been shown that it practically *only* occurs at the stated places in the soil. In some more recent experiments of HANSEN'S, vigorous growths of the ferment in well-closed Chamberland filter-tubes were placed below the surface of the earth. Three years later, the contents of the tubes were introduced into sterilised wort, and a vigorous development of the ferment was obtained. Its life period may thus extend beyond a year.

Finally, it remained to be proved that the soil is its *true* habitat during the winter; in order to prove this, HANSEN examined dust from the most diverse places from January to June, also the dried fallen fruits of many plants, and finally various excrements. His seventy-one analyses gave a negative result, and thus furnished the proof that *the true winter habitat of the ferment is the soil under the fruit trees.* It retains its ordinary appearance during the long winter-time, and in the summer it is again carried into the air by the united action of insects and wind, and, through these two means of transport, it is further distributed from fruit to fruit.

It is evident that at the time when the ferment appears in abundance on the ripe fruits mentioned, it may also be carried by the wind to surrounding objects and so on to unripe fruits. Even in his first memoir, HANSEN stated that *the rare occurrence on unripe fruits* might be due to the ferment *quickly perishing, partly from want of nourishment and partly from the drying up of its cells.* He subsequently proved by experiment the correctness of this view. He stirred up with water old and young cells, and placed them in thin layers, either on object-glasses or on tufts of cotton-wool well teased out, which were then allowed to dry under protection from the sun. In less than twenty-four hours all the cells had perished. It is self-evident that isolated cells lying on unripe fruit are still more unfavourably placed than in the experiment. If, however, thick layers of the cells are wrapped in cotton-wool or filter-paper, they will continue to live for a long time, as they do in the earth—in filter-paper, for instance, for more than eight months.

No complete investigations have been published on the life-history of other alcoholic ferments. *Saccharomycetes occur very generally on fruits containing a sweet juice.* For several years HANSEN has carried on experiments similar to those described, with species of *Saccharomycetes* which often occur in fruit gardens, such as *Sacch. Pastorianus I., Sacch. ellipsoideus I.*, also with Carlsberg bottom-yeast No. 1, and with some top-fermentation beer-yeasts. He always found that yeasts *sown in the soil in September were alive a year later.* Some species had formed spores at the surface of the soil.

The wine-yeasts belonging to the group *Saccharomyces*

ellipsoideus kept alive in the earth for more than three years.

Thus, it has been proved, that these fungi may also winter in the earth. Whether this is their only mode of circulation in nature, has not as yet been ascertained.

In antithesis to these direct observations of HANSEN, is PASTEUR'S view that the wine-yeasts are *un*able to live in the soil from one season to the next. Where the yeasts come from which are found on grapes at the time of ripening, PASTEUR was unable to say.—MUELLER-THURGAU arrived at the same results as HANSEN.

MYCODERMA CEREVISIÆ AND VINI.

It is characteristic of these species that they very readily form films on various alcoholic liquids. Under these names

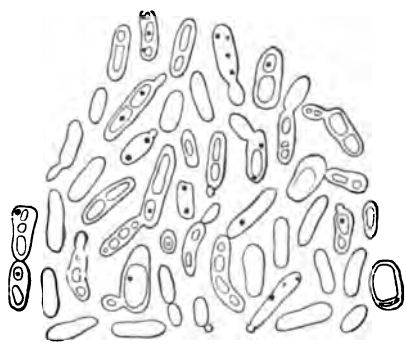

FIG. 79.—Mycoderma cerevisiæ from Copenhagen breweries. (HOLM.)

are included a number of different species, some of which may excite a feeble alcoholic fermentation; they behave differently towards lager beer, some causing disease whilst others do not.

The *Mycoderma cerevisiæ* (Fig. 79) examined by HANSEN, which is very generally met with in Copenhagen breweries, forms variously-shaped cells. The cells are usually transparent and less refractive than the true *Saccharomycetes*; to each cell there are generally one, two, or three highly refractive particles, which often have a quivering, rolling motion. This micro-organism forms a dull, greyish, wrinkled film on wort and beer,

and does not excite alcoholic fermentation; neither does it invert solutions of cane-sugar.

The colonies on the surface of the gelatine are *light grey, dull,* and *spread out like a film* or *hollowed like a shell.* By means of this macroscopic appearance *Mycoderma* is readily distinguished from the ordinary *Saccharomycetes,* which, on the same medium, form light greyish-yellow colonies with a dry or lustrous surface, and a more or less arched form. *Sacch. membranæfaciens,* which differs so markedly in its biological behaviour, and which very rapidly gives a strong film on the liquid, alone resembles *Mycoderma* in its behaviour on plate cultures.

The form of film described was obtained by HANSEN when lager beer had been exposed in open vessels at temperatures between 2° and 15° C.; at 33° C. development still occurred, but at temperatures above 15° C. this species gave place more and more to competing forms. As low temperatures are favourable to its development, it will readily thrive in the storage cellar, especially as lager beer forms a much more favourable medium for its growth than wort. This is seen to be the case when traces of a pure film are introduced into lager beer and wort, contained in open vessels, and then left to develop; the culture in lager beer nearly always remains pure, while in wort various other species make their appearance.

In HANSEN'S comprehensive series of experiments on Carlsberg beer, it was always found that both lager and export beers were attacked by this fungus; but there was never the slightest indication that the beer had acquired any disease from this source. The fungus was widely distributed just at those periods when the beer was found to be particularly stable and of good flavour. This has also been confirmed by numerous experiments on lager and export beers conducted by GROENLUND and A. PETERSEN, and those carried out in the author's laboratory. It is self-evident that we are only speaking of beer which has been properly treated. In imperfectly closed bottles and casks, *Mycoderma cerevisiæ* will of course rapidly develop a film, which is sufficient, unaided, to destroy the product.

BÊLOHOUBEK was the first to find that, under certain con-

ditions, *Mycoderma* may cause considerable injury in the brewery. Subsequently, KUKLA described a curious cloudiness in lager beer, having the appearance of a cloud of fine dust in the liquid, which manifested itself either during storage or after tapping; he attributes this disease to *Mycoderma*, and further assumes that it is weak wort, having certain peculiarities in its composition, which specially favours the development of *Mycoderma*. It is to be hoped that further investigations will throw more light on this subject.

HANSEN expressed the opinion that the name *Mycoderma cerevisiæ* denotes not one, but several different species, and LASCHÉ'S experiments subsequently confirmed this. The latter investigator describes four different species which he isolated from cloudy beers. They are distinguished from the species described by HANSEN by the fact that they produce alcohol in beer-wort; one yields 0·26 per cent. by volume, two yield 0·79 per cent., and the fourth produces 2·51 per cent. LASCHÉ concludes from his experiments that these four species cause diseases in beer, both turbidity and changes in taste and odour; in this respect they also differ from HANSEN'S *Mycoderma*. LASCHÉ is inclined to assume that the chemical composition of the wort has no influence on the disease caused by *Mycoderma*, for, in his experiments, the disease was produced in worts of high extract and worts of low extract, in worts rich in sugar and worts poor in sugar.

It is frequently stated that the chemical activity of certain species of *Mycoderma*, on the surface of vinous liquids, is a process of oxidation by which alcohol is converted in some cases into carbon dioxide and water, in others into acetic acid; higher fatty acids are also said to be formed, and these may be converted into ethereal salts (SCHULZ).[1]

LAFAR discovered a budding fungus resembling *Mycoderma*, in the cask-sediment of a lager beer, which produces acetic acid fermentation. The developed film of this species exhibits the typical appearance of the corresponding growth-forms of *Mycoderma cerevisiæ*.

WINOGRADSKY found that the *Mycoderma* occurring on *wine*, prepared in pure culture by HANSEN'S method, *alters its shape*

[1] Ad. Mayer, *Lehrbuch der Gärungschemie*, p. 213, 1879.

with the composition of the nutritive solution; he experimented both with solutions, the mineral constituents of which remained constant while the organic substances varied, and also with solutions in which the reverse was the case.

In experiments with pure cultures of *Mycoderma vini*, WORTMANN found that this fungus is capable of injuring the *taste* of wine long before it has formed a visible film; on the other hand, at this stage, the bouquet of the wine is not impaired to any considerable extent.

Although DE SEYNES, REESS, ENGEL, and CIENKOWSKI claimed to have found ascospores in *Mycoderma*, it has not since been possible to bring about this formation. It would appear from the figures given that the fat globules, which occur in many unicellular fungi during the resting stage, had been mistaken for spores; in some cases the mistake appears to have arisen through the presence of an admixture of true *Saccharomycetes*. The old name *Mycoderma* is therefore more appropriate to this fungus than the new term *Saccharomyces*.

CHAPTER VI.

THE APPLICATION OF THE RESULTS OF SCIENTIFIC RESEARCH IN PRACTICE.

THE actual fermentation process plays a very important part in all branches of the fermentation industries. The better insight which has been gradually acquired into the process has been rendered possible by the development of the science of the fermentation organisms. This development may be divided into three historic periods.

The investigations of the *first* period all relate to the important question, whether living organisms can come into existence by *spontaneous generation.* The *second* period is especially distinguished by PASTEUR'S classical researches. In the *third* period, dating from 1879, founded by HANSEN, a practical reform was carried out for the first time.

1. During the first period (1745 to 1857) the *theory of sterilisation and disinfection was established, and its application in practice was assured.*

SPALLANZANI'S discoveries in connection with spontaneous generation formed not only the starting-point of modern bacteriology, but also acquired great importance in practice. In 1782, SCHEELE proved that vinegar may be preserved unchanged after it has been heated, and APPERT (1810) likewise showed that beer, wine, and other liquids can be preserved by similar treatment. It was further shown that air can be purified by passing it through a strongly-heated tube (SCHWANN) or through a cotton-wool filter (SCHROEDER and DUSCH). From this the conclusion was drawn that water

can be purified by a similar process, provided the filter is sufficiently dense.

The theory of the use of *antiseptics* was scientifically established as early as 1839, when SCHWANN published his discovery that yeast-cells are killed by the action of certain chemicals, and that fermentation may be brought to a complete standstill by such treatment.

2. The PASTEUR period dates from 1857. The great service performed by this investigator was his proof that bacteria exert an influence on different fermentations, and that they can produce diseases in liquids which are undergoing alcoholic fermentation. The necessary practical deductions lead to attempts, for example, to prevent the access of impure air to the liquids. The consequence—as regards the brewery—is the abandonment of open coolers and refrigerators, the aëration of the wort by air which has been previously sterilised, and the purification of air in the fermenting rooms.

The statements in Chapter VII. of PASTEUR'S "Études sur la bière" (1876), regarding the importance of the *oxidation of wort* during cooling, should also be alluded to. By means of direct determinations of the amount of oxygen in wort, PASTEUR showed that a certain quantity of oxygen, partly in the free state and partly combined in the wort, exerts an influence on the course of the fermentation and on the clarification, but that when the proportion of oxygen in the wort exceeds certain limits it may act injuriously on the character (*force et arome*) of the beer (page 377).

Although several investigators have undertaken elaborate researches in this direction, it has not hitherto proved possible to establish any fixed rules for practical guidance. These must be determined by trial experiments for each individual case.

SCHEELE and APPERT'S method for the treatment of vinegar, wine, and beer, at high temperatures, was taken up by PASTEUR, and through his great authority obtained a wide application (the so-called Pasteurising). Milk has recently been treated in this manner, more especially since KOCH proved that the tuberculosis bacillus is so widely distributed.

The experiments on *aëration* described in "Études sur

la bière" gave rise to extensive series of investigations which yielded valuable information on the *fermentative* and *reproductive* power of yeast in the presence of varying amounts of air. This relation plays an important part in the *distillery* and in *pressed-yeast works.* No results, however, have as yet been obtained which can be directly applied in practice.

The reason why the method proposed by PASTEUR for the purification of yeast has acquired no real importance for practical purposes, has been already stated.

3. With HANSEN'S investigations on the alcoholic ferments there began, as AUBRY says, a new era in the history of the fermentation industries. His earliest publications on this subject date back to 1879. In the year 1883 he demonstrated that the universally dreaded yeast-turbidity and the disagreeable changes in taste and odour, in fact some of the commonest and worst *diseases of beer*, are not caused by bacteria, by the water, by the malt, by the particular method of brewing, etc., as was then commonly believed, but that these diseases *must be attributed to the yeast itself*; for in such cases the pitching-yeast contains, in addition to the cultivated species, *other Saccharomycetes, which act as disease germs* (*Sacch. Pastorianus I. and III., Sacch. ellipsoideus II.*). *A basis was thus acquired for the new system.*

He subsequently showed that the *name Saccharomyces cerevisiæ embraces many different races or species* (*both bottom- and top-fermentation*), which communicate very different characters to beer.

As the rational result of these scientific investigations he completed the cycle of his new system by his method for the *pure cultivation of yeast.* If it were possible to free the impure yeast-mass from wild yeasts as well as from bacteria and mould-fungi, we should still not attain all we desire; for *if the purified yeast contains several species of Saccharomyces cerevisiæ, we are still dealing with a mixture which is just as uncertain as before the purification,* and, moreover, *the composition of such a yeast-mass is always liable to change during fermentation.* In fact, HANSEN has shown in recent investigations that cases occur in which *two yeasts, each of which by*

itself gives a faultless product, will, when mixed, give rise to disease in the beer. He made these experiments with the two species of Carlsberg bottom-yeast No. 1. and No. 2; in one set of experiments the pitching-yeast consisted chiefly of No. 1 with a small admixture of No. 2, and in the other set, the reverse was the case. It was found that in all cases the small quantity of the admixed yeast, whether No. 1 or No. 2, made the beer less stable as regards yeast-turbidity, than when the chief constituent of the pitching-yeast was employed alone. Thus the two cultivated yeasts under these conditions behaved in such a manner as to produce effects similar to those brought about by the wild yeasts (*Sacch. Pastorianus III.* and *Sacch. ellipsoideus II.*). We can therefore only obtain true *uniformity in working* when *a suitable species* has been obtained from the yeast-mass *by systematic selection, and has been cultivated by itself.*

The systematic studies which HANSEN has carried on for many years on the *constancy of the characters of different species of yeast*, have proved that, under the conditions of the brewery, they commonly only undergo slight changes, which are of no importance in practice, and this result has been confirmed by various investigators.

On the other hand, he found that, when the life conditions of the yeast are disturbed by a systematic and more vigorous treatment, it was possible *to produce varieties*, which remained more or less constant in their properties, and even to produce new species. As a result of these investigations HANSEN obtained useful varieties of some brewery yeasts.

The biological and physiological characteristics of the species discovered by HANSEN led him also to a *method for the practical analysis of brewery yeast*, by means of which it is possible to ensure in time against the prevalence of foreign yeasts. It was proved by his experiments on a large scale that the forms which produce yeast-turbidity may be present to the extent of one part in forty-one of the pitching-yeast, and the species (*Sacch. Pastorianus I.*) which produces a disagreeable odour and an objectionable bitter taste to the extent of one part in twenty-two, without exercising any injurious influence, provided the brewing operations are conducted under

the *yeast-propagating apparatus* devised by HANSEN and KUEHLE, which, when once charged with an absolutely pure cultivation, will work continuously for years. The apparatus (Fig. 80) is described in detail with directions for use in HANSEN'S "Practical Studies in Fermentation"; it consists of three principal parts with the connecting tubes, viz.: (i.) the arrangement for aërating the wort, consisting of the air-pump (*A*) and air-vessel (*B*); (ii.) the fermenting-cylinder (*C*), and (iii.) the wort-cylinder (*D*).

The air, which is partially purified by previous filtration, is pumped into the air-vessel, from which it can be passed to the wort-cylinder or to the fermenting-cylinder. In both cases it has to pass through sterilised cotton-wool filters (*g*, *m*). The *wort-cylinder* is connected by piping directly with the copper, from which the boiling hot hopped wort is run into it; it is then aërated in the closed cylinder and cooled by water from *p*. The wort is then forced into the *fermenting-cylinder*, which, like the wort-cylinder, is constructed on the same principle as the ordinary two-necked flask. It is fitted with a doubly-bent tube (*c*, *d*), which dips into a vessel containing water; a vertical glass tube (*f*, *i*, *f*) for measuring the height of the liquid in the cylinder; an appliance (*b*, *b*) for stirring up the settled yeast, and a cock (*l*) for drawing off the beer and the yeast. At about the middle of the cylinder there is a small side tube (*j*), fitted with india-rubber connection, pinch-cock, and glass-stopper. When a portion of the wort has been forced into the fermenting-vessel, the pure yeast—which is forwarded to the brewery in a flask specially constructed for this purpose—is introduced through the rubber tube at *j*; this is again closed, and the remainder of the wort may then be added either at once or after the lapse of a few days, according to the quantity of yeast which has been introduced.

Where it is necessary to regulate the temperature during fermentation, the fermenting-vessel is surrounded by a water-jacket.

By means of this simple apparatus it is possible to obtain, at short intervals, absolutely pure pitching-yeast sufficient for about eight hectolitres of wort. As already stated, the apparatus, when once started, works continuously. For further

details the reader is referred to the exact description in HANSEN'S work.

A modification of the propagating apparatus has been devised by BERGH and JOERGENSEN (Fig. 81). The filtered air

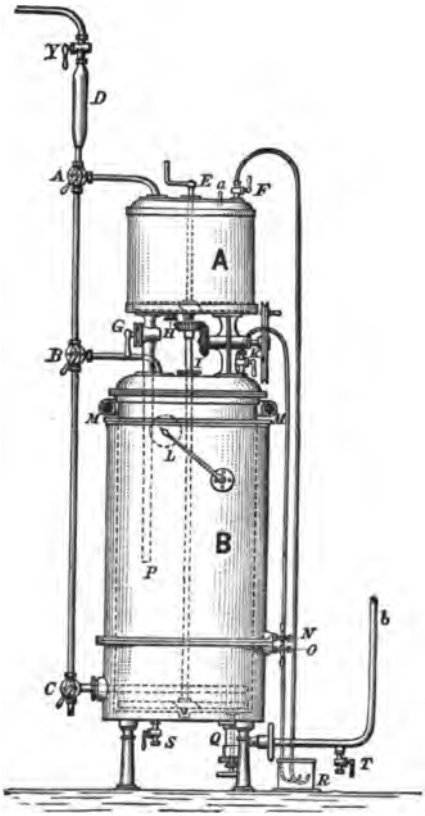

FIG. 81.—Yeast-propagating apparatus devised by BERGH and JOERGENSEN.

passes through the three-way cocks at *A*, *B*, and *C*, into the two cylinders **A** and **B**. The upper cylinder holds about 50 litres, and the lower cylinder 160 litres. **A** is provided with a stirrer (*E*), a tube (*a*) for introducing the yeast and withdrawing samples. The bent tube *F* is for the exit of the carbon dioxide. The tube *G P* connects the two cylinders, and the connection can be made or broken by means of the cock *G*. *H* is the outlet for the water used in cleaning *A*.

The cylinder **B** is surrounded by a cast-iron jacket made in two parts; the upper portion serves as a water-jacket for cooling the wort and for regulating the fermentation; the lower portion is used as a steam-jacket, and is provided with a cock at *O* for the entrance of the steam, and another at *S* for the outlet. *M* is a ring-shaped tube provided with small holes; this is connected with the cold-water main during the cooling of the wort; the water flows out at *N*. The stirrer *J* is set in motion by means of toothed gear. The height of the liquid in the cylinder is indicated by means of a float, connected with which is a pointer and arc *L*. Connected with the top of the cylinder is the bent tube *K*. At the bottom is the cock *Q*, which is in connection with the pipe *b*. Both the bent tubes dip into the vessel *R*, which is filled with water.

The wort is introduced into the lower cylinder, where it is treated in the ordinary manner. The pure culture is introduced into the upper cylinder, and is then washed down into the lower cylinder by means of a little wort, which is forced from **B** into **A**, and then back again into **B**. When a vigorous multiplication of the yeast has set in, the liquid is stirred up, and a portion forced into **A**; this is to be used to start the next fermentation. The cylinder **B** thus serves alternately as fermenting-cylinder and wort-cylinder.[1]

Other modifications have been devised by BROWN and MORRIS, ELION, KOKOSINSKI, and VAN LAER; more widely different are the forms devised by P. LINDNER and MARX.[2]

[1] Both the forms of apparatus described above are manufactured by Messrs. BURMEISTER and WAIN, of Copenhagen; HANSEN and KUEHLE'S apparatus is also made by W. E. JENSEN, of Copenhagen.

[2] An apparatus which has now become of considerable importance as part of HANSEN'S system of pure yeast cultivation is the *closed cooler* mentioned above, by means of which it is possible to introduce the wort into the fermenting-vessel absolutely pure and properly aërated. Appliances having this for their object were devised by VELTEN shortly after the publication of Pasteur's *Études*, and were constructed in accordance with PASTEUR'S theoretical views, but at that time they could not acquire any practical importance; for what was the use of having a pure wort when the disease-germs were again introduced with the yeast? The construction of the Velten apparatus was also rather objectionable, incandescent tubes being used for sterilising the air. A model construction is found, on the contrary, in the *Old Carlsberg apparatus*, in which the air is sterilised through cotton-wool. The conditions which render

In order to be able to send to a distance the selected *pure cultures in a liquid condition*, special forms of flasks were devised by HANSEN (Fig. 82) and by the author (Fig. 83). The yeast can be sent to great distances in these flasks, and there is no difficulty in safely transferring it from the flask to the cylinder of the propagating apparatus.

FIG. 82.

FIG. 83.

In sending small quantities of pure cultures, in such a manner that they may be safely and readily employed for further cultivation, the small *Hansen flasks* are employed. They are connected, in the flame, with the Pasteur flask in which the pure culture has developed. A trace of the yeast is transferred to the cotton-wool, and the flask is again closed in the flame with the asbestos stopper, which is then coated over with sealing-wax. When the culture is to be used, the flask is

such appliances useful are now attained for the first time through the introduction of pure yeast, and open coolers will therefore gradually disappear in the future.

again connected with a Pasteur flask containing wort, and the yeast is rinsed into the latter.

This method has proved valuable for sending absolutely pure yeast to tropical countries, rendering it possible to send large collections of pure cultivations to Australia, South America, and the most distant Asiatic countries at small expense.[1]

It is of the greatest importance to note that, even after the lapse of years, *the particular yeast once selected can always be procured again*, a sample of the pure culture being preserved in the laboratory in a 10 per cent. solution of cane-sugar. In such a solution cultivated yeasts can be kept alive for years.[2]

The absolutely pure and systematically selected races of yeast, prepared in large quantities for industrial purposes, are now employed in numerous breweries in all beer-producing countries, not only in Europe, but also in America, Asia, and Australia. HANSEN made his first experiment in 1883 in the well-known Old Carlsberg brewery at Copenhagen.

Since HANSEN'S *system* was first carried out in a bottom-fermentation brewery, it naturally found its first application in breweries of the same kind, and in these it has reached the highest degree of perfection. *Any one who wishes to become acquainted with the application of this system* to one or other branch of the fermentation industries, should therefore *take, as the starting-point of his studies, the results achieved in bottom-fermentation brewing.*

The first pure cultivated and methodically selected *high-*

[1] The use of sterilised filter-paper for sending yeast samples is for quite a different object. This method is used for sending an impure brewery yeast to a laboratory in order that a pure culture may be prepared from it.

[2] It is not advisable to preserve ferment-organisms on gelatines. Thus P. FRANKLAND observed that fermentation bacteria may lose their fermentative power more or less completely if cultivated for a considerable length of time on a solid medium; sometimes the fermentative power disappeared after one single cultivation. The same result was obtained by NENCKY with regard to certain lactic acid bacteria. The writer has shown that high-fermentation yeasts in use in breweries lose some of their most valuable practical properties by cultivation on wort-gelatine, for cultures developed from such materials clarified less readily and effected too active a fermentation.

fermentation yeast types were introduced into a brewery by the author in the year 1884. It was urged, as an objection to their adoption, that, in consequence of the high temperatures at which such fermentations are conducted, the yeast would not long remain pure. Experience has proved that this objection is of no moment, and that in this section of the brewing trade important progress may be made and considerable advantages realised by the use of a single *selected* type. More recently, it has been stated that it is impossible to secure a *suitable after-fermentation with one species*—an objection which had previously been erroneously urged in the case of low-fermentation yeast. VAN LAER especially has laid stress upon this supposed disadvantage; and, while admitting that there exist *low*-fermentation yeasts capable of carrying through a normal after-fermentation, he has asserted that the writer wrongly attributed the same properties to high-fermentation yeasts. In spite of our accurate experiments, and although practical results had been obtained with a single *selected* species, even in English breweries, to which VAN LAER'S theory (unsupported as it was by any *exact* evidence) especially related, our experience was disregarded, and he prepared *mixtures* of *high-fermentation yeasts* for use in breweries, which, he claimed, are able to fulfil all practical requirements. Thus the one species would carry out the principal, the other the secondary fermentation. Indeed, the possibility of preparing such a "composite yeast" cannot be denied; but assuming VAN LAER'S preparations yielded satisfactory results in the breweries, it does not follow that these are due to the action of the "composite yeast" as such. By no means; it had first to be proved that this new yeast was really a *composite* yeast in *practice, i.e.* that the different species of which it was composed were really able to co-operate. In conjunction with J. CHR. HOLM, the author examined several of the preparations sold for use in breweries, with the result that, even during the first fermentation, one of the species prevailed at the expense of the others, the latter entirely disappearing during the following fermentations. Thus, it was shown that the problem of preparing a real "composite yeast" had not been solved. The experience gained in recent years, even in English breweries,

has invariably confirmed the correctness of our first results, viz., in high- as well as in low-fermentation the whole fermentation can be carried through with a single selected type of yeast.

HANSEN'S epoch-making investigations of the last decade have given rise to a very extensive literature, much of which records original work of great value, throwing light on his system from many sides, and thus facilitating both a true conception of its importance and its practical application.

It would lead us too far to discuss all the principal points embodied in the literature. In concluding this description, we will confine ourselves to a few quotations from the highest authorities, those who have contributed to the elucidation of our subject and to the extended adoption of the system in different countries.

Professor C. LINTNER gives the following review of the situation in 1885:—[1]

"Now that different breweries have employed pure cultivations of the Carlsberg yeasts, and that the Scientific Station at Munich has also introduced pure cultivations of Munich yeasts into various breweries, the results obtained may be summarised as follows:

"1. By contamination with so-called wild yeasts, a brewery yeast, normal in other respects, may gradually become incapable of producing a beer of good flavour and with good keeping properties.

"2. A contamination of this kind can occur through wild yeasts present in atmospheric dust during summer and autumn, or the wild yeast may be introduced with the pitching-yeast or with cask sediment.

"3. By means of HANSEN'S methods of analysis and pure cultivation, it is possible to isolate from a contaminated yeast the desired brewery yeast in a good and pure condition.

"4. The pure cultivated yeast possesses in a marked degree the properties of the original yeast previous to

[1] *Zeitschrift f. d. ges. Brauw.*, 1885, p. 399.

contamination, both as regards the degree of attenuation, and the taste and keeping properties of the beer.

"5. Different races of normal bottom-fermentation yeast (*Sacch. cerevisiæ*) exist with specific properties, which are constant for each race and form distinctive characteristics."

Professor AUBRY, Director of the Scientific Brewing Station at Munich, wrote (1885)[1]:—"In addition to the breweries mentioned (Spatenbräu and Leistbräu in Munich), a large number of breweries at home and abroad have carried out experimental fermentations with pure Carlsberg yeast. The results which were expected were naturally not attained in all cases, the degree of attenuation was found to be too low in the greater number of cases,[2] the taste was not the one popular in the locality, etc., etc., but *all the reports which reached us were favourable as regards the keeping properties, brilliance, and the freedom of the beer from any taste of yeast.* The good properties of the yeast have brought about its permanent introduction into many breweries, as, for instance, the Liesinger brewery, at Liesing, near Vienna. In the present brewing season the Spaten brewery in Munich has made extensive use of yeast obtained from Carlsberg, and a great part of the pitching-yeast used in the brewery of the Franziskanerkeller in Munich, during the winter, was also derived from pure cultures of Carlsberg yeast. The course of fermentation and the results with regard to the taste, condition, and keeping properties of the beer, answered all requirements. The property of giving a somewhat low attentuation appears to be characteristic of the yeast, for it remains constant. The taste of the beer at first differs somewhat from the ordinary Munich taste, but approaches more nearly to this with later generations; it remains, however, soft and agreeable."

Dr. WILL, of the Scientific Brewing Station, Munich, writes (1885):[3]—"If now, as I trust I have made clear, it is possible to detect with certainty the species of yeast which have an injurious influence in the brewery, we must make practical

[1] *Zeitschrift f. d. ges. Brauw.*, 1885.

[2] Carlsberg bottom-yeast No. 2, a quick clarifying species.

[3] *Allgem. Brauer- und Hopfenzeitung*, 1885.

use of this knowledge, and only employ pitching-yeasts which do not show the characteristics mentioned above for injurious species which so frequently and actively exert a disturbing influence in the brewery. This, however, will only be possible when yeast-cells endowed with the properties of normal bottom-yeast are isolated from the ordinary brewing yeast, and further cultivated with the exclusion of every contamination; in other words, *when only pure cultivated yeast* is employed in the brewery. HANSEN is entitled to the greatest praise in this particular direction, since he has pointed out a way and devised a method which enabled him to attain the desired end. The far-reaching results which were obtained in Old Carlsberg with pure cultivated yeast have already caused many other breweries to employ only pure cultivated yeast, and the results in general have given satisfaction when varieties of normal bottom-yeast were chosen which corresponded with the requirements as regards attenuation and taste.

"It is to be hoped, therefore, that the value of pure cultivated yeast may be recognised in ever-increasing circles, and many old prejudices regarding the yeast overcome; also that the smaller breweries, which have besides many difficulties to contend with, will not resist the conviction that a number of calamities may be avoided by the introduction of pure cultivated yeast into an otherwise well-conducted brewery. The amount expended will yield a liberal interest."

Dr. REINKE, who is at the head of the experimental brewing station of the Royal Agricultural College in Berlin, made the following significant statement of the situation in 1888:—[1]

"Without the exact study of HANSEN'S pioneer investigations, and without their utilisation, no one at the present time is able to permanently survive the competition in the brewing industry. HANSEN'S researches have brought about a revolution in the brewery, especially with regard to the treatment of the yeast."

Professor BÊLOHOUBECK, of the Bohemian Polytechnic at Prague, says in his well-known biography of Hansen, 1889:[2]—

[1] *Chemiker-Zeitung*, 29 Dec., 1888, p. 1749.

[2] *Zeitschrift f. d. ges. Brauw.*, Munich, 1889, p. 505.

"No one will be surprised that the establishment of the principle of pure yeast-cultivation, and the truly crushing criticism concerning the general custom of leaving fermentations in the brewery to chance which until then prevailed, and, above all, that the actual introduction into the brewery of pure cultivated yeast prepared by HANSEN'S method, produced at first astonishment amongst practical men—with some honourable exceptions—then ridicule, and finally provoked hostile opposition; for it is known to the initiated what obstinate conservatism there is in brewing circles, where all innovations and reformatory efforts are not only met with passiveness and mistrust, but are sometimes most tenaciously resisted. Fortunately many important factors were united in the struggle against the opposition, which finally suffered a decided defeat in spite of the support of some theoretical specialists, more particularly in North Germany and Austria-Hungary. It was chiefly the correctness of HANSEN'S views which contributed to this victory, and which completely convinced the most eminent authorities of Europe of the science of fermentation; secondly, the fact that able experts outside Denmark also began to experiment with pure yeast-cultivation; thirdly, the highly favourable results which were obtained in the brewery with pure yeast; and, finally, the fact that in 1887 Professor HANSEN, in conjunction with Captain KUEHLE, succeeded in devising a pure yeast apparatus which enabled them to produce large quantities of the pure yeast which had been prepared on a comparatively small scale in the laboratory. At the present time hundreds of breweries obtain a pure cultivated standard yeast from institutions in which pure cultivations of beer-yeast are prepared, and thousands of breweries do the same indirectly in that they obtain their pitching-yeast from the above breweries. *The universal employment of pure yeast in the brewing industry is now therefore only a question of time.*

"If we now weigh with the most complete objectiveness the significance of these facts as applied to the conditions obtaining in European bottom-fermentation breweries, we are compelled to acknowledge that the reform introduced by

Professor HANSEN is still more far-reaching than is generally assumed. A result of this reform is already being discussed in brewing circles, namely, the abandonment of open coolers in all breweries where pure yeast is employed, as these freely permit of the contamination of the wort with micro-organisms, and especially with bacteria and the so-called 'wild' yeasts. It is therefore proposed to filter the hopped wort, or to separate the suspended matter (cooler-deposits) by another method, to saturate the wort with filtered air, and to cool it by artificial means. But these are by no means all the precautions which must be adopted in order to guard against further infection of the wort in the fermenting-rooms and in the lager-cellar. Only when these questions have been solved—perhaps by means of closed fermenting-vessels of a suitable material, by the sterilisation of the fermenting and storage vessels, by a more rational arrangement of the fermenting-rooms and lager-cellars, and by the ventilation of these by means of filtered air, etc.—only then will it be possible amongst beer producers and consumers to enjoy to the full the great advantages of having a beer of better quality and keeping properties than the present beer—advantages which are a result of the employment of pure yeast.

"The above statements concerning the importance of pure cultivated yeast refer throughout to beer bottom-yeast only. There could be no doubt even from the first that it would also be possible to employ HANSEN'S method of pure yeast-culture to top-fermentation yeast, and with the same result; this has since been proved experimentally by ALFRED JOERGENSEN, and pure top-yeast has proved just as successful in the brewery as pure bottom-yeast. The writer of these lines is convinced that the introduction of pure cultivated species of yeast into distilleries, and especially into pressed-yeast factories, will give very advantageous results. In distilleries—other conditions being maintained the same—better fermentations and a greater yield of alcohol in comparison with the average now attained are to be expected, whilst in pressed-yeast factories a better yield of yeast should result from a successful selection of a pure cultivated species, and possibly the employment of clear mashes will then be

found preferable to mashes containing the grains as now employed."

In Dr. H. BUNGENER'S treatise "La levure de la bière," 1890,[1] the following statement occurs, contrasting the old with the new period:—"In France, HANSEN'S system has been eagerly taken up by L. MARX, A. FLUEHLER, and KOKOSINSKI. In some breweries, it has been recently introduced, and it will soon be adopted by others. We are convinced that its introduction into all the larger breweries of France, and in fact everywhere else, will only be a question of time. It has in fact been established, that it ensures regular working and a good result in one of the most important stages of the manufacture, where hitherto chance and, consequently, uncertainty also prevailed."

Prof. C. J. LINTNER, of the Technical College, Munich, writes (1891)[2]:—"In the abstracts relating to advances in the brewing industry, the epoch-making investigations of the Danish *savant* EMIL CHR. HANSEN, and their application in breweries, have been frequently reported. A connected account of HANSEN'S reform and methods, however, has not yet appeared in this journal, though such an account would be by no means undesirable, considering the great importance which the subject has acquired during the seven years since its introduction into the brewing industry. Hitherto, the brewery has mainly benefited from HANSEN'S system, which, however, has already found its way into the distillery and pressed-yeast factory, and these branches of the fermentation industry will also be greatly benefited by its introduction."

In England some of the most celebrated authorities have frankly acknowledged the value of HANSEN'S investigations. Amongst these is Professor PERCY FRANKLAND, who has expressed himself as follows:—[3]

"EMIL CHRISTIAN HANSEN, of Copenhagen, has *enormously extended our knowledge of the alcohol-producing organisms or yeasts*; he has shown that there are a number of distinct

[1] *Moniteur scientifique du Dr. Quesneville*, Juillet-Août, Paris, 1890.

[2] *Dingl. Polytechn. Journal*, Jahrg. 72, Bd. 279, Heft 9.

[3] Royal Institution of Great Britain. Meeting, February 19, 1892.

forms, differing indeed but little amongst themselves in shape, but possessing very distinct properties, more especially in respect of the nature of certain minute quantities of secondary products to which they give rise, and which are highly important as giving particular characters to the beers produced. HANSEN has shown how these various kinds of yeast may be grown or cultivated in a state of purity even on the industrial scale, and has in this manner revolutionised the practice of brewing on the continent. For during the past few years these pure yeasts, each endowed with particular properties, have been grown with scrupulous care in laboratories equipped expressly for this purpose, and these pure growths are thence despatched to breweries in all parts of the world, particular yeasts being provided for the production of particular varieties of beer. In this manner scientific accuracy and the certainty of success are introduced into an industry in which before much was a matter of chance, and in which nearly everything was subordinated to tradition and blind empiricism."

In *top-fermentation* the system was founded on the theoretical and practical studies of the author, and has now been introduced into all countries. After its adoption by various American and Australian breweries, worked on the English system, W. R. WILSON succeeded (1892) in carrying out this important reform in a London brewery (Messrs Combe & Co.), both primary and secondary fermentation being effected by a single selected species of yeast. According to the reports in English journals numerous breweries in Great Britain have successfully adopted HANSEN'S system of pure cultivated yeast.

The following is taken from a report by J. C. MACCARTIE of Melbourne:—[1]

"The Burton yeast[2] yields a mild 'round' flavoured beer of great brilliancy and stability, and one that is excellently suited for bottling. I now come to a matter that should be of interest to your readers. Mr. DE BAVAY and I read with some astonishment the statements made by certain scientific gentle-

[1] *The Brewers' Journal*, 1889, No. 291, p. 489.

[2] A "Burton" species obtained in pure cultivation in the author's laboratory from English yeast.

men in England, concerning the difficulty or impossibility of obtaining after or secondary fermentation when *one* type of yeast alone—say *Sacch. cerevisiæ*—is used; for there has not been the slightest difficulty in obtaining secondary fermentation in 'stock' or bottled ales, where the Australian or 'Burton' yeasts have been used here.

"I have, with Mr. DE BAVAY, over and over again examined both 'stock' and bottled ales fermented with pure Burton yeast, and that secondary fermentation was vigorous in them, any one who saw the foam and 'head' on the beers could not doubt.

"Mr. DE BAVAY tells me that he frequently obtains a well-marked secondary cask-fermentation a fortnight after racking the beer, and this in cases where the yeast used was fresh from the laboratory, and therefore practically free from the slightest intermixture of other types of yeast." (DE BAVAY'S brewery in Melbourne is worked on the top-fermentation system.)

MACCARTIE, therefore, basing his opinion on these facts, has no doubt but that within a few years HANSEN'S system will also be adopted in all the important *top-fermentation* breweries of the world. His detailed account is of great interest, since it affords fresh proof, obtained in actual practice, that differences exist in the species of *Saccharomyces cerevisiæ*, and thus it also proves the necessity for making a selection from these species with reference to practical requirements.

Dr. E. KOKOSINSKI, Director of the laboratory at Lille, writes:—[1]

"In August, 1888, after three years' preparatory study, I introduced pure cultivated yeast for the first time into a top-fermentation brewery in Lille. Shortly afterwards, at the beginning of 1889, I also introduced it into some other breweries in Lille, Roubaix, Douai, and St. Omer, and at the present time there are fifteen breweries in North France in which pure yeast is employed, and *all, without a single exception, obtain excellent results with it.*"

[1] "Application industrielle de la méthode Hansen à la fermentation haute dans le Nord de la France," *Compt. rend. de la Station scientifique de Brasserie*, Gand, 1890.

He summarises the results of his *practical experience with beers obtained with the help of pure cultivated top-yeast*, as follows:—

"1. They have the particular flavour which the brewer wishes to obtain;

"2. This taste is uniform and always remains constant; it is characterised by great pureness;

"3. The clarification takes place more readily and more quickly;

"4. The beers are more resistant to the action of bacteria and have a greater stability.

"It follows from the above that if HANSEN'S method has rendered great service in bottom-fermentation, it is now about to do the same also for top-fermentation, and in the latter case it will be of far greater value, for in top-fermentation we have not the advantage of the low temperature which obtains in bottom-fermentation, and which tends to check the action and the development of disease germs."

W. R. WILSON says, in the "Brewers' Journal" (London, 1892, p. 527):—

"Some gyles have been prepared with pure yeast, and have been compared against ale brewed at the same time, but pitched with ordinary yeast. It is admitted on all hands that, so far as can be at present ascertained, the *pure-yeast ale* is *immeasurably superior* to the other. HANSEN'S *pure yeast system* applied to English high fermentations appears to be *equally suitable* for *ales*, *porters*, and *stouts*."

Since then the pure yeast system has been completely carried out in Combe & Co.'s brewery, and, according to reports in English professional journals, many English brewers have employed the species selected by WILSON with uniform success.

Similar opinions on the importance of HANSEN'S work have been expressed by other zymotechnologists such as DELBRUECK (Berlin), FLUEHLER (Lyon), GRIESSMAYER (Munich), LANGER (Mödling), PRIOR (Nürnberg), MAERCKER (Halle), MARX (Marseilles), SCHWACKHOEFER (Vienna), THAUSING (Vienna), and others. Several of his former opponents have become his warmest supporters.

In the above we have only spoken of the brewing industry.[1] HANSEN'S discoveries have, however, already been applied to other branches of industry in which alcoholic fermentation plays a part. Thus experiments have been made at many places in *pressed-yeast factories* and in *distilleries*, and in a great number the system has already been introduced with success. An important observation made by DELBRUECK and others may be mentioned, viz., that an increase in the yield of alcohol was often observed in distilleries in which pure yeasts were used, without any considerably better attenuation. This may be accounted for on the assumption that foreign ferments which consume a part of the nutrient liquid, without producing alcohol, are suppressed by the use of the absolutely pure culture.

In *wine-fermentation* a beginning was made, in 1888, by a pupil of HANSEN'S, L. MARX, of Marseilles; subsequently other investigators have worked in the same direction in France, MUELLER-THURGAU in Switzerland, FORTI and PICHI in Italy, MACH and PORTELE in Austria, and WORTMANN in Germany. NATHAN (Wurtemberg) and KAYSER (France) have likewise made extensive experiments in connection with the fermentation of fruit-wines.

The author has had frequent occasion in the course of years to point out to the industries concerned the advantages offered by the use of selected types of yeast both in the manufacture of grape-wine and in that of fruit- and berry-wine. A specially

[1] The "natural" pure culture of yeast or yeast-culture proposed by DELBRUECK, which was to be performed in the breweries themselves in order to free their yeast from wild yeast, is based simply on certain isolated observations, *e.g.* that certain wild yeast species in competition experiments were observed to develop more freely at lower temperatures than the culture-yeasts employed in the same experiments. It stands to reason, however, and has been proved by experience, that no general rule can be formulated from such detached observations. Neither is it possible by means of the processes recommended by DELBRUECK, but known and used long before in breweries, such as pumping the wort immediately after fermentation has begun into other vessels, or pitching with the beer when the head forms on the surface at the moment fermentation sets in ("Kräusen"), to obtain certain results with regard to the suppression of foreign ferments, still less in relation to the selection of the best type of culture-yeast. Nor has DELBRUECK'S suggestion acquired any practical significance—as might have been foreseen.

interesting instance of the energetic action of certain types of wine-yeast is supplied by the extensive production, in the Scandinavian countries, of wine prepared from *raisin* juice but fermented with genuine wine-yeast.

In addition to the quotations made above, the following statement by Professor J. WORTMANN, director of the pure yeast-culture station of the German Association for Viticulture at Geisenheim, may be given :—

"If, with a view of throwing light on the general results obtained in practice, we sum up the main contents of the reports, we shall find at once that wherever pure yeast can be employed in such a manner as to be without a rival from the very beginning—in other words, wherever the desired fermentation can easily be carried out in practice by the exclusive use of the pure yeast added—a favourable effect of the pure yeast is in all such cases unmistakable. This is especially true of the fermentations carried out in the preparation of sparkling wine, and of the renewed fermentations of wines. But in these cases we also find—and this point is most important—that we are in the right track regarding the method adopted for the use of the yeasts, since it has led to such good results even in these first experiments.

"Further, we are sure, judging from the results so far secured, to obtain satisfactory effects with pure yeasts in all those cases in which musts of little value or fruit- and berry-musts are fermented. The yeasts present in fruit- and berry-musts are presumably apiculate or other dangerous forms, and are soon overcome by a vigorous pure yeast, but apart from this we have it in our power to utilise the type of yeast adopted to the best advantage, for owing to the repression of the bouquets peculiar to such musts, the peculiarity of the pure yeast will become more prominent, especially with regard to bouquet-formation.

"In the fermentation of grape-musts with pure yeasts, owing to the fact that yeasts, contained in the musts themselves, are very vigorous and efficacious, under the circumstances we can only expect to secure success if it is possible to bring the pure yeast into the must in such a manner that it predominates at the outset over the yeasts already present. This is a *conditio*

sine qua non. The results of practical experiments have clearly proved that this is possible.[1]

"We now know from experience gained in the course of many years that the suppression of the numerous varieties of organisms existing in must and originating from berry-skins—mould-fungi, wild yeasts, *Mycoderma*, and bacteria—frequently leads to a radical improvement of the fermentation-products, and that the suppression of those detrimental organisms may be effected by yeasts of different types, that is, definite action—in other words, we know that the beneficial results referred to can be secured by the use of pure yeast in the fermentation of grape-, fruit-, and berry-juices. This improvement in smell and taste of the wine often manifests itself during fermentation, and at all events after it has ceased. Moreover, the beneficial effect of pure yeast does not end here. For during fermentation the pure yeast has not only asserted its fermentative activity, but has also checked, if not entirely prevented, the growth of the entire crowd of organisms occurring spontaneously in the must and originating from the berry-skins, among which organisms unsuitable yeasts and even directly disease-producing species, such as *Mycoderma*, acetic-acid bacteria, etc., are never wanting. Thus, *not only the must, but also the fermentation-product, the wine, in all its further stages,* is *subject to the domination of the pure yeast.* Hence, *the beneficent influence* of the pure yeast will not only make itself felt during the period immediately following the end of the principal fermentation, but it will continue. This is likely to be specially advantageous to the development of wine in bottles owing to the pure yeast present exercising a repressive influence on other organisms. It may be added that all experience capable of exact control, gained since the introduction of pure yeast into practice in wine-manufacture, goes to prove the *great* and *lasting influence* of *pure* yeast on the fermentation-product."[2]

[1] "On the Experience hitherto obtained in Practice regarding Pure Yeasts." A paper read at the thirteenth German Congress of Viticulture at Maintz, 1894. See also WORTMANN'S very instructive treatise, "The Use and Effect of Pure Yeasts in the Manufacture of Wine," Berlin, 1895.

[2] "On Artificial After-fermentations of Wines in Bottle and Cask," *Landwirtschaftl. Jahrb.*, 1897.

One result of HANSEN'S epoch-making discoveries has been the establishment of *special laboratories*, the object of which is to prepare absolutely pure material for employment in practice, to carry out control analyses, and to instruct the younger generation in the true understanding and the proper application of his discoveries. Such institutions have now been established in almost all countries, and are partly private and partly supported by the State; they have already produced a number of able teachers, analysts, and technologists, who are working with energy and judgment to propagate more widely, in science and practice, the views of the Danish investigator.

HANSEN'S work has exercised an indirect influence on *dairy* methods; as mentioned in a previous chapter, pure cultures of lactic-acid bacteria have been successfully employed on a large scale for the souring of cream. Finally, also, in the *tobacco fermentation*, SCHLOESING and SUCHLAND have experimented with the view of producing a definite aroma in tobacco leaves by the addition, during the fermentation, of pure cultures of certain species of bacteria.

The idea which underlies all these efforts at reform is the principle which has been recognised and carried out for centuries in horticulture and agriculture, namely, that in order to obtain the desired species of plant its seed should be sown free from the seed of all other plants.

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